A Thesis for the degree of Doctor of Philosophy

Genetic and pathogenic characterization of classical swine fever virus LOM field strains isolates from Jeju island

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List of Abbreviations

A	adenine
aa	amino acid
α-MEM	alpha-minimum essential medium
CAVAC	ChoongAng Vaccine Laboratories
CSFV	classical swine fever virus
CSS	clinical significance score
Ct	cycle threshold
CV	commercial vaccine
DPI	days post-inoculation
END	exaltation of Newcastle disease virus
FBS	fetal bovine serum
IM	intramuscular
IN	intranasal
INDEL	insertion-deletion
LOM	low virulent strain of Miyagi isolate
MLV	modified live vaccine
NSP	non-structural protein
nt	nucleotide
OIE	the World Organization for Animal Health (the Office of International Epizootics)
ORF	open reading frame
PCV2	porcine circovirus 2
PI	persistently infected
PRRSV	porcien reproductive and respiratory syndrome virus
RACE	rapid amplification of cDNA ends
SDM	standard deviation of the mean difference
SE	swine erysipelas
SL	stem-loops
SPF	specific pathogen-free
SS	single-stranded intervening sequences
$TCID_{50}$/ml	50% tissue culture infectious dose per milliliter
U	uridine
UTR	untranslated region

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Chapter 1

Genotypic characterization of classical swine fever (CSF) virus LOM field isolates from Jeju island

1. Abstract

After the unintentional vaccination of the LOM vaccine strain in 2014, classical swine fever virus (CSFV) reemerged in naïve pig herds on Jeju Island, South Korea, which had been a CSF-free region with a non-vaccination policy for a decade. Since the re-emergence, endemic outbreaks of CSFV have occurred in Jeju, causing enormous damage to provincial pig farms. The present study reports the complete genome sequences and molecular characterization of the LOM-derived field CSFV strains responsible for the current outbreaks on Jeju Island. The emergent Jeju LOM-derived isolates shared 98.9–99.7% and 98.7–99.0% nucleotide sequence identity at the E2-gene and whole-genome levels compared to the LOM vaccine strain, respectively. Genetic and phylogenetic analyses indicated that the CSFV field isolates were closest to the LOM strains, but appeared to have undergone substantial evolution. The total number of nucleotide and amino acid differences between the LOM vaccine strain and LOM-derived field isolates ranged from 111 and 28 to 148 and 42. These variations were found to be widely distributed throughout the genome and particularly accumulated in non-structural proteins, which might be associated with the potential for LOM to revert to its original low pathogenic form and subsequent horizontal transmission in Jeju swine herds. These data improve our knowledge regarding safety of the LOM vaccine and inherent risk of reversion to natural virulence in host animals.

Key words: CSFV; Endemic outbreaks; Jeju Island; LOM-derived field strains; Reversion

2. Introduction

Classical swine fever (CSF) is a highly contagious multisystemic viral disease of pigs that economically affects the global swine industry, and it is listed as a notifiable disease by the World Organization for Animal Health (OIE) (OIE, 2014). The disease was first recognized in the United States in 1810, and then, rapidly spread worldwide (Edwards et al., 2000). Although it has been successfully eradicated from several countries, endemic or sporadic outbreaks continue to occur in most pig-producing countries in Asia, South America, and Eastern Europe (Blome et al., 2017). The causative agent of CSF is the classical swine fever virus (CSFV), a small enveloped RNA virus belonging to the *Pestivirus* genus of the family *Flaviviridae*, together with bovine viral diarrhea virus and border disease virus (Lindenbach et al., 2007). CSFV has a single-stranded, positive-sense RNA genome of approximately 12.3 kb with one large open reading frame (ORF) that is flanked by 5′- and 3′-untranslated regions (UTRs). The single ORF encodes a polyprotein 3898 amino acids in length, that undergoes co- and post-translational proteolysis by cellular and viral proteases to eventually produce the 12 processing products N^{pro}, C, E^{rns}, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Ji et al., 2015) (Figure 1).

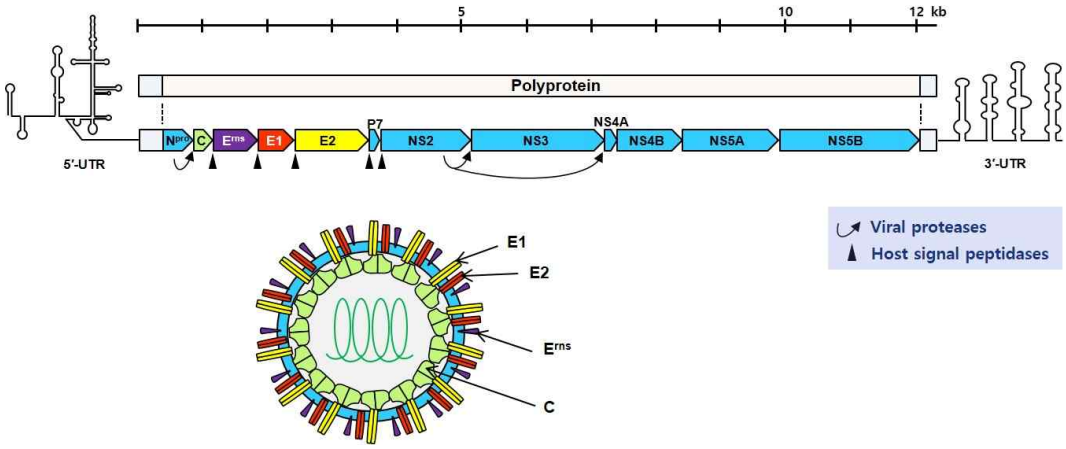

Figure 1. Schematic diagram of CSFV virion structure and genome organization. CSFV has a single-stranded, positive-sense RNA genome of approximately 12.3 kb long and contains one large open reading frame (ORF) that is flanked by 5′- and 3′-untranslated regions (UTRs). The single ORF encodes a encodes one precursor polyprotein that undergoes co- and post-translational proteolysis by viral and cellular proteases to produce the 12 mature products N^{pro}, C, E^{rns}, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B.

Most countries, except those in the European Union, adopt a vaccination policy using modified live vaccines (MLVs) to prevent and control CSF (Ji et al., 2015). The LOM virus was initially derived from a low virulent strain of Miyagi isolate from Japan and was attenuated through continuous propagation in bovine kidney cell culture (Nishimura et al., 1964; Sato et al., 1969). It was further cloned to select the exaltation of Newcastle disease virus (END) phenomenon-negative (END−) strain from the attenuated LOM strain (END+) to improve the safety and immunogenicity in South Korea (Choi et al., 1988). The attenuated LOM strain has been solely used nationwide as a live vaccine to eradicate CSFV, and its safety and efficacy has been proven in pigs for several decades (Kim et al., 2008). Despite mandatory vaccination with the LOM strain, sporadic CSF outbreaks have occurred in mainland South Korea (Kim et al., 2008; Yoo et al., 2018). In contrast, the national government-driven CSF eradication program has succeeded in Jeju Province, the largest island in South Korea that is 80-km and 304-km away from the mainland at the closest and farthest points, respectively. Consequently, Jeju Island banned vaccination in 1998 and became a CSF-free region in 1999, and since then, had maintained a CSFV-naïve status for over a decade (Song et al., 2013). However, CSFV simultaneously re-emerged on Jeju Island in 2014 in areas with extensive pig production. Clinical presentations include reproductive failures such as abortions and stillbirth in pregnant sows and cutaneous hyperemia or cyanosis, watery diarrhea, and death in young pigs (Je et al., 2018). Genetic and phylogenetic analyses revealed that the re-emergent CSFV isolates have >99% identity with the LOM vaccine strain (Je et al., 2018). Epidemiological investigations concluded that the CSFV vaccine was

introduced into Jeju Island through the accidental distribution of a CSF-swine erysipelas (SE) combined live vaccine. Since 2014, LOM outbreaks have been confirmed every year in dozens of pig farms on the island, thereby tremendously threatening the unvaccinated livestock of the provincial pork industry. The aims of this study is to determine the complete genome sequences of the LOM-derived CSFV field isolates responsible for the ongoing outbreaks on Jeju Island and investigate the potential relationship of genetic variation and virulence.

3. Materials and Methods

3.1. Clinical sample collection

Continuous CSFV outbreaks occurred in several non-vaccinated farms in the Hallim area of Jeju province, and the affected animals varied in the ages, including fetuses, pre- and post-weaning piglets, and growing pigs, and their mortality rates. Clinical samples of aborted fetuses and dead pigs that suffered from severe diarrhea and atrophy before dying from five different unvaccinated swine farms during 2017−2018 outbreaks were independently submitted for laboratory analysis (Table 1). Tissue specimens including tonsil, lymph node, spleen, and lung were taken from each carcass, and all samples were prepared into 10% suspensions as described previously (Lee et al., 2015).

Table 1 2017−2018 CSF cases from which the LOM-derived strains were identified in this study

	Incidence type (year)	Ages of affected pigs	Sample sizes	Clinical manifestation	Abortion or mortality rate[a]
Farm A (KNU-1823)	New occurrence (2017)	Pregnant sows (50−60 days of pregnancy)	1	Abortion	8%
Farm B (KNU-1824)	Recurrence[b] (2017)	Pregnant sows (100−110 days of pregnancy)	1	Abortion	4%
Farm C (KNU-1825)	Recurrence[c] (2018)	Suckling and weaned piglets (5−30 days)	3	Atrophy, diarrhea, and death	50%
Farm D (KNU-1826)	Recurrence[d] (2018)	Growing pigs (40 days)	4	Atrophy, diarrhea, and death	10%
Farm E (KNU-1827)	New occurrence (2018)	Suckling piglets (10−20 days)	2	Death	50%

[a]The rate in the corresponding age group

[b]The first LOM confirmed in 2015

[c]The first LOM confirmed in 2016

[d]The first LOM confirmed in early 2018

3.2. Nucleotide sequence analysis

The full-length genomic sequences of the 2017−2018 Jeju LOM isolates from the clinical samples and the LOM strain from a commercial vaccine (CV) were determined via a traditional Sanger sequencing method. Seven overlapping cDNA fragments encompassing the entire viral genome were RT-PCR amplified using oligonucleotide primers synthesized based on the published LOM sequence (GenBank accession number EU789580), and newly amplified isolate sequences and the individual PCR products were sequenced as described previously (Lee et al., 2015). The 5′ and 3′ ends of the viral genome were also determined via rapid amplification of cDNA ends (RACE) as described previously (Lee and Lee, 2013). The sequence data of all LOM-derived field isolates have been deposited in GenBank under accession numbers MK093241 through MK093252.

3.3. Multiple alignments and phylogenetic analyses

The complete sequences of the 50 E2 genes and 46 genomes of global CSFV strains were independently used in sequence alignment and phylogenetic analyses. Multiple sequence alignments were generated with the ClustalX 2.0 program (Thompson et al., 1997) and the percentages of nucleotide sequence divergences were further assessed using the same software program. Phylogenetic trees were constructed from the aligned nucleotide or amino acid sequences by using the neighbor-joining method and subsequently subjected to bootstrap analysis with 1000 replicates to determine the percentage reliability values of each internal node of the tree (Saitou and Nei, 1987). All Figures involving phylogenetic trees were generated using Mega 4.0 software (Tamura et al., 2007).

4. Results and discussion

Like LOM vaccine strains, all Jeju LOM isolates, designated as KNU-1823 to -1827, identically consisted of 12,298 nucleotides (nts), which are composed of a 373-nt 5′-UTR, a 3898-amino acid (aa) coding region, and a 228-nt 3′-UTR (Figure 2). The similarity between the E2 protein genes was initially measured, and the sequence homology and the number of nt/aa differences are described in Table 2. Nucleotide sequence analysis showed high homology among the Jeju LOM isolates, ranging from 98.8 to 100% and 98.9 to 100% identities at the nucleotide and amino acid levels, respectively. Similarly, all emergent LOM isolates shared high identities with the previous Jeju isolates (JJ-1601 and -1602) and LOM vaccine strains, showing 98.9 to 99.7% and 99.1 to 100% aa identities, respectively. Although the number of aa differences consistently ranged from 2−5 between the LOM vaccine strain and the Jeju LOM isolates, the number of nt substitutions significantly increased in 2018, implicating the potential for further additional genetic drift in the field.

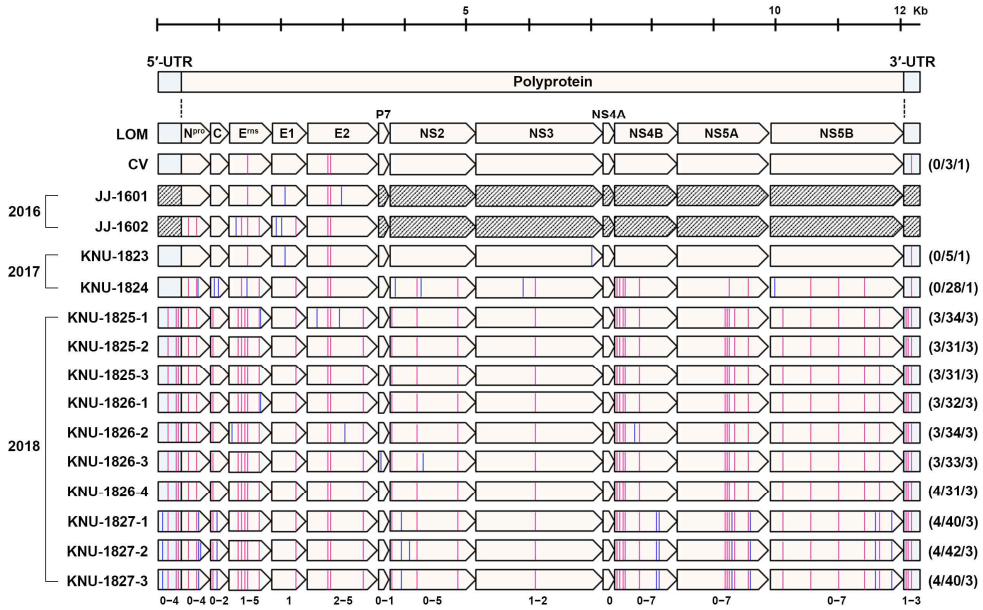

Figure 2. Genetic variations in the CSFV LOM-derived field strains responsible for endemic outbreaks on Jeju Island. Schematic representation of amino acid (aa) differences between the reference LOM vaccine strain and the 2016–2018 isolates. The illustration on top represents the organization of the CSFV genome that is composed of a 5′-UTR, a single polyprotein (N^{pro}, C, E^{rns}, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B), and a 3′-UTR. The vertical lines represent 1-aa substitution in relation to the aa sequence of the reference LOM strain. The red lines indicate common substitutions found in more than two isolates, whereas the blue lines show unique mutations identified in each isolate. The slashed boxes on JJ-1601 and JJ-1602 represent unavailable sequences in the GenBank database. The digits within parenthesis on the right indicate the number of individual differences in the 5′-UTR, polyprotein, and 3′-UTR, respectively, compared to the reference LOM vaccine strain. The digits on the bottom indicates the minimum to the maximum number of changes in each UTR and protein.

Table 2 Pairwise comparisons of the E2 protein genes of the LOM-derived and reference LOM strains

Strain	Nucleotide/amino acid identity (%) (No. of nucleotide/amino acid differences)															
	LOM	CV	JJ-1601	JJ1602	1823	1824	1825-1	1825-2	1825-3	1826-1	1826-2	1826-3	1826-4	1827-1	1827-2	1827-3
LOM/1980		99.8 (2)	99.5 (5)	99.4 (6)	99.6 (4)	99.5 (5)	98.8 (13)	99.1 (10)	99.1 (10)	99 (11)	99.1 (10)	99.1 (9)	99.1 (10)	98.9 (12)	98.9 (12)	98.9 (12)
Commercial vaccine (CV)	99.4 (2)		99.7 (3)	99.6 (4)	99.8 (2)	99.7 (3)	99 (11)	99.2 (8)	99.2 (8)	99.1 (9)	99.2 (8)	99.3 (7)	99.2 (8)	99.1 (10)	99.1 (10)	99.1 (10)
JJ-1601	99.1 (3)	99.7 (1)		99.5 (5)	99.5 (5)	99.4 (6)	98.7 (14)	99 (11)	99 (11)	98.9 (12)	99 (11)	99.1 (10)	99 (11)	98.8 (13)	98.8 (13)	98.8 (13)
JJ-1602	99.4 (2)	100 (0)	99.7 (1)		99.4 (6)	99.7 (3)	99.1 (9)	99.4 (6)	99.4 (6)	99.3 (7)	99.4 (6)	99.5 (5)	99.4 (6)	99.2 (8)	99.2 (8)	99.2 (8)
KNU-1823	99.4 (2)	100 (0)	99.7 (1)	100 (0)		99.5 (5)	98.8 (13)	99.1 (10)	99.1 (10)	99 (11)	99.1 (10)	99.1 (9)	99.1 (10)	98.9 (12)	98.9 (12)	98.9 (12)
KNU-1824	99.4 (2)	100 (0)	99.7 (1)	100 (0)	100 (0)		99.1 (10)	99.3 (7)	99.3 (7)	99.2 (8)	99.3 (7)	99.4 (6)	99.3 (7)	99.1 (9)	99.1 (9)	99.1 (9)
KNU-1825-1	98.6 (5)	99.1 (3)	98.9 (4)	99.1 (3)	99.1 (3)	99.1 (3)		99.5 (5)	99.5 (5)	99.8 (2)	99.5 (5)	99.6 (4)	99.5 (5)	99.3 (7)	99.3 (7)	99.3 (7)
KNU-1825-2	99.1 (3)	99.7 (1)	99.4 (2)	99.7 (1)	99.7 (1)	99.7 (1)	99.4 (2)		99.8 (2)	99.7 (3)	99.8 (2)	99.9 (1)	100 (0)	99.6 (4)	99.6 (4)	99.6 (4)
KNU-1825-3	99.1 (3)	99.7 (1)	99.4 (2)	99.7 (1)	99.7 (1)	99.7 (1)	99.4 (2)	100 (0)		99.7 (3)	99.8 (2)	99.9 (1)	99.8 (2)	99.6 (4)	99.6 (4)	*99.6 (4)*
KNU-1826-1	99.1 (3)	99.7 (1)	99.4 (2)	99.7 (1)	99.7 (1)	99.7 (1)	99.4 (2)	100 (0)	100 (0)		99.7 (3)	99.8 (2)	99.7 (3)	99.5 (5)	99.5 (5)	99.5 (5)
KNU-1826-2	98.9 (4)	99.4 (2)	99.1 (3)	99.4 (2)	99.4 (2)	99.4 (2)	99.1 (3)	99.7 (1)	99.7 (1)	99.7 (1)		99.9 (1)	99.8 (2)	99.6 (4)	99.6 (4)	99.6 (4)
KNU-1826-3	99.1 (3)	99.7 (1)	99.4 (2)	99.7 (1)	99.7 (1)	99.7 (1)	99.4 (2)	100 (0)	100 (0)	100 (0)	99.7 (1)		99.9 (1)	99.7 (3)	99.7 (3)	99.7 (3)
KNU-1826-4	99.1 (3)	99.7 (1)	99.4 (2)	99.7 (1)	99.7 (1)	99.7 (1)	99.4 (2)	100 (0)	100 (0)	100 (0)	99.7 (1)	100 (0)		99.6 (4)	99.6 (4)	99.6 (4)
KNU-1827-1	99.1 (3)	99.7 (1)	99.4 (2)	99.7 (1)	99.7 (1)	99.7 (1)	99.4 (2)	100 (0)	100 (0)	100 (0)	99.7 (1)	100 (0)	100 (0)		100 (0)	100 (0)
KNU-1827-2	99.1 (3)	99.7 (1)	99.4 (2)	99.7 (1)	99.7 (1)	99.7 (1)	99.4 (2)	100 (0)	100 (0)	100 (0)	99.7 (1)	100 (0)	100 (0)	100 (0)		100 (0)
KNU-1827-3	99.1 (3)	99.7 (1)	99.4 (2)	99.7 (1)	99.7 (1)	99.7 (1)	99.4 (2)	100 (0)	100 (0)	100 (0)	99.7 (1)	100 (0)	100 (0)	100 (0)	100 (0)	

The percent nucleotides identity was shown in the upper right, and the percent of amino acid was presented in the lower left.

The genome of a representative strain from a commercial vaccine had almost identical 99.9% homology with that of a reference LOM vaccine strain at both the nt and aa levels, resulting from 1-aa, 2-aa, and 1-nt variations in E^{rns}, E2, and 3′-UTR, respectively. However, except for KNU-1823 which is mostly identical to vaccine strains rather than field isolates, most Jeju LOM isolates had nt (98.7–99.0%) and aa (98.9–99.2%) identities with the LOM vaccine strain at the genomic level. The total number of nt/aa differences ranged from 111/28 to 148/42, and these differences were found to be widely distributed throughout the genome and particularly accumulated in NS2, NS4B, NS5A, and NS5B (Figure 2). In addition, the 2018 isolates contained 3–4-nt and 3-nt variations in the 5′- and 3′-UTRs, respectively. The number of nt/aa differences and the percentage identity shared between the Jeju isolates and the reference LOM vaccine strain are summarized in Table 3. Compared to the complete LOM vaccine genome, the KNU-1823, -1824, -1825, -1826, and -1827 genomes showed a 14-nt (99.8% homology), 111-nt (99.0% homology), 127–135-nt (98.9% homology), 131–139-nt (98.8–98.9% homology), and 145–148-nt (98.7–98.8% homology) difference, resulting in 5, 28, 31–34, 31–34, and 40–42 non-silent point mutations, respectively (1 in E^{rns}, 1 in E1, 2 in E2, and 1 in NS3 for KNU-1823; 3 in N^{pro}, 2 in C, 3 in E^{rns}, 1 in E1, 2 in E2, 4 in NS2, 2 in NS3, 5 in NS4B, 2 in NS5A, and 4 in NS5B for KNU-1824; 2 in N^{pro}, 1 in C, 5–6 in E^{rns}, 1 in E1, 3–5 in E2, 3 in NS2, 1 in NS3, 5 in NS4B, 5 in NS5A, and 5 in NS5B for KNU-1825; 2 in N^{pro}, 1 in C, 5–6 in E^{rns}, 1 in E1, 3–4 in E2, 0–1 in P7, 3–4 in NS2, 1 in NS3, 5–6 in NS4B, 5 in NS5A, and 5 in NS5B for KNU-1826; 3–4 in N^{pro}, 2 in C, 5 in E^{rns}, 1 in E1, 3 in E2, 4–5 in NS2, 1 in NS3, 7 in

NS4B, 7 in NS5A, and 7 in NS5B for KNU-1827). Subsequent phylogenetic analysis based on the complete E2 protein sequence clearly distinguished the CSFV strains into three genotypes (1, 2, and 3) with representative subgroups for each genotype (Figure 3a). All the Jeju LOM-derived field strains belonged to the subgroup 1.1, which clustered closely around the previous Jeju isolates and vaccine strains. In whole-genome phylogeny, the 2017−2018 Jeju strains grouped within the same cluster as the LOM vaccine strains (Figure 3b). These data suggest the evolutional distance between the LOM vaccine strains and LOM-derived field isolates. Altogether, our results indicate that the emergent Jeju LOM-derived CSFV isolates are still highly homologous to the LOM vaccine strains, but undergo continuous genetic mutations that might be involved in reverting to its natural low virulent phenotype and responsible for the recent outbreaks on Jeju Island.

Table 3 Comparison of nucleotide (nt) and amino acid (aa) sequence identities of LOM-derived field isolates with LOM vaccine strain

Strain	Nucleotide/amino acid identity (%) (No. of nucleotide/amino acid differences)														
	5′ UTR	N^{pro}	C	E^{rns}	E1	E2	P7	NS2	NS3	NS4A	NS4B	NS5A	NS5B	3′ UTR	Total
CV	100 (0)	100 (0)	100 (0)	99.8/99.5 (1/1)	100 (0)	99.7/99.4 (3/2)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	99.5 (1)	99.9/99.9 (4/3)
JJ-1601	NA[a]	99.8/100 (1/0)	99.6/100 (1/0)	99.5/99.5 (3/1)	99.4/99.4 (3/1)	99.5/99.1 (5/3)	NA	NA	NA	NA	NA	NA	NA	NA	NA
JJ-1602	NA	99.4/98.8 (3/2)	99.6/100 (1/0)	98.5/98.2 (10/4)	99.1/98.4 (5/3)	99.4/99.4 (6/2)	NA	NA	NA	NA	NA	NA	NA	NA	NA
KNU-1823	100 (0)	100/100 (0/0)	100/100 (0/0)	99.8/99.5 (1/1)	99.8/99.4 (1/1)	99.6/99.4 (4/2)	100/100 (0/0)	99.9/100 (1/0)	99.7/99.8 (5/1)	100/100 (0/0)	99.9/100 (1/0)	100/100 (0/0)	100/100 (0/0)	99.5 (1)	99.8/99.8 (14/5)
KNU-1824	100 (0)	98.6/98.2 (7/3)	98.9/97.9 (3/2)	98/98.6 (13/3)	98.8/99.4 (7/1)	99.5/99.4 (5/2)	96.1/100 (8/0)	99.1/99.1 (12/4)	99.2/99.7 (15/2)	99.4/100 (1/0)	99.1/98.5 (9/5)	99.1/99.5 (13/2)	99.2/99.4 (17/4)	99.5 (1)	99/99.2 (111/28)
KNU-1825-1	99.1 (3)	98.4/98.8 (8/2)	98.6/98.9 (4/1)	97.9/97.3 (14/6)	98.9/99.4 (6/1)	98.8/98.6 (13/5)	96.6/100 (7/0)	99.3/99.3 (9/3)	99.3/99.8 (14/1)	99.4/100 (1/0)	98.6/98.5 (14/5)	98.7/98.9 (18/5)	99/99.3 (20/5)	98.6 (3)	98.9/99.1 (134/34)
KNU-1825-2	99.1 (3)	98.6/98.8 (7/2)	98.6/98.9 (4/1)	97.9/97.7 (14/5)	98.9/99.4 (6/1)	99.1/99.1 (10/3)	96.6/100 (7/0)	99.1/99.3 (11/3)	99.5/99.8 (10/1)	100/100 (0/0)	98.6/98.5 (14/5)	98.7/98.9 (18/5)	99/99.3 (20/5)	98.6 (3)	98.9/99.2 (127/31)
KNU-1825-3	99.1 (3)	98.6/98.8 (7/2)	98.6/98.9 (4/1)	97.9/97.7 (14/5)	98.8/99.4 (7/1)	99.1/99.1 (10/3)	96.6/100 (7/0)	99.1/99.3 (11/3)	99.3/99.8 (14/1)	99.4/100 (1/0)	98.5/98.5 (15/5)	98.7/98.9 (18/5)	99/99.3 (21/5)	98.6 (3)	98.9/99.2 (135/31)
KNU-1826-1	99.1 (3)	98.6/98.8 (7/2)	98.6/98.9 (4/1)	97.9/97.3 (14/6)	98.9/99.4 (6/1)	99/99.1 (11/3)	96.6/100 (7/0)	99.3/99.3 (9/3)	99.2/99.8 (15/1)	99.4/100 (1/0)	98.5/98.5 (15/5)	98.7/98.9 (18/5)	99/99.3 (20/5)	98.6 (3)	98.9/99.1 (133/32)
KNU-1826-2	99.1 (3)	98.6/98.8 (7/2)	98.6/98.9 (4/1)	97.7/97.3 (15/6)	98.8/99.4 (7/1)	99.1/98.9 (10/4)	96.6/100 (7/0)	99/99.3 (13/3)	99.2/99.8 (15/1)	100/100 (0/0)	98.4/98.2 (16/6)	98.7/98.9 (18/5)	99/99.3 (21/5)	98.6 (3)	98.8/99.1 (139/34)
KNU-1826-3	99.1 (3)	98.6/98.8 (7/2)	98.6/98.9 (4/1)	97.9/97.7 (14/5)	98.9/99.4 (6/1)	99.1/99.1 (9/3)	96.1/98.5 (8/1)	99.1/99.1 (12/4)	99.3/99.8 (14/1)	100/100 (0/0)	98.6/98.5 (14/5)	98.7/98.9 (18/5)	99/99.3 (20/5)	98.6 (3)	98.9/99.1 (132/33)
KNU-1826-4	99.1 (3)	98.6/98.8 (7/2)	98.6/98.9 (4/1)	97.9/97.7 (14/5)	98.9/99.4 (6/1)	99.1/99.1 (10/3)	96.6/100 (7/0)	99.1/99.3 (11/3)	99.3/99.8 (14/1)	100/100 (0/0)	98.6/98.5 (14/5)	98.7/98.9 (18/5)	99/99.3 (20/5)	98.6 (3)	98.9/99.2 (131/31)
KNU-1827-1	98.9 (4)	98.6/98.2 (7/3)	98.3/97.9 (5/2)	97.7/97.7 (15/5)	98.6/99.4 (8/1)	98.9/99.1 (12/3)	97.1/100 (6/0)	98.8/99.1 (16/4)	99.1/99.8 (18/1)	99.4/100 (1/0)	98.4/97.9 (16/7)	98.7/98.5 (19/7)	99.1/99 (18/7)	98.6 (3)	98.7/98.9 (148/40)
KNU-1827-2	98.9 (4)	98.2/97.6 (9/4)	98.3/97.9 (5/2)	97.9/97.7 (14/5)	98.6/99.4 (8/1)	98.9/99.1 (12/3)	97.1/100 (6/0)	98.8/98.9 (16/5)	99.1/99.8 (18/1)	99.4/100 (1/0)	98.4/97.9 (16/7)	98.7/98.5 (19/7)	99.2/99 (17/7)	98.6 (3)	98.7/98.9 (148/42)
KNU-1827-3	98.9 (4)	98.6/98.2 (7/3)	98.3/97.9 (5/2)	97.9/97.7 (14/5)	98.6/99.4 (8/1)	98.9/99.1 (12/3)	97.1/100 (6/0)	98.9/99.1 (15/4)	99.1/99.8 (17/1)	99.4/100 (1/0)	98.4/97.9 (16/7)	98.7/98.5 (19/7)	99.1/99 (18/7)	98.6 (3)	98.8/98.9 (145/40)

[a]NA, Not available.

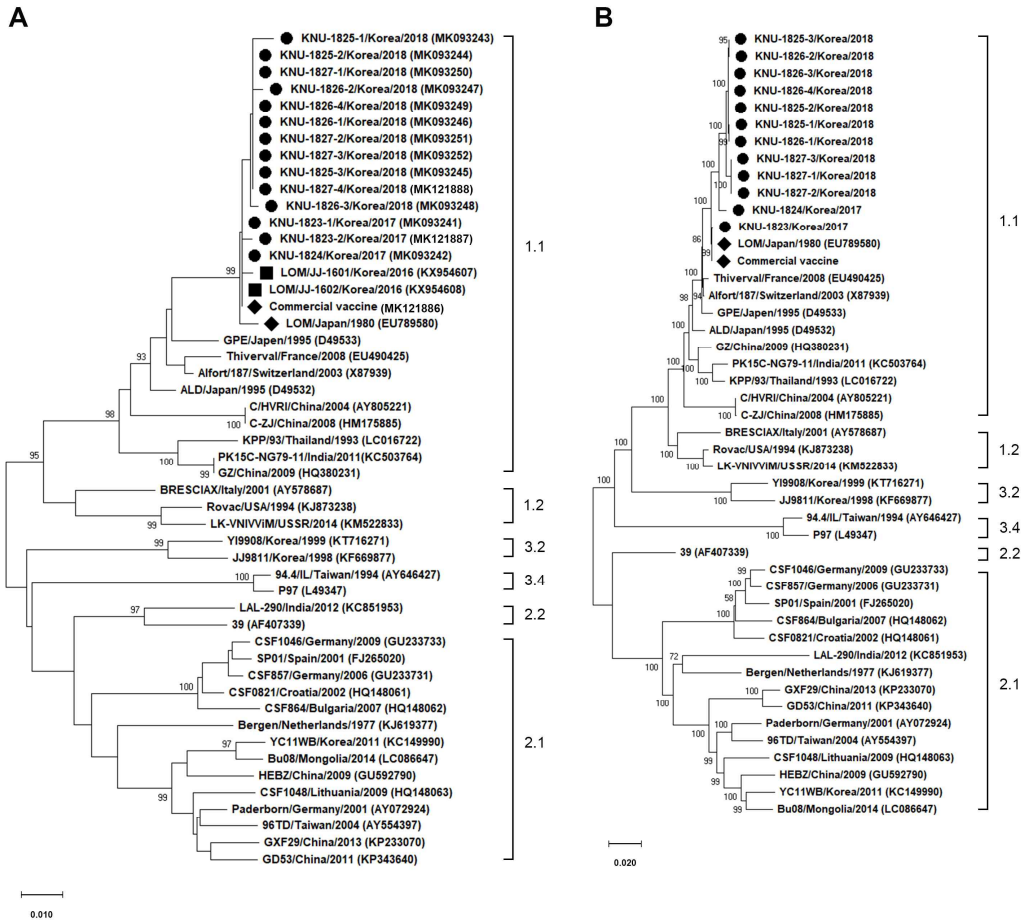

Figure 3. Phylogenetic analyses based on the nucleotide sequences of the E2 genes (a) and full-length genomes (b) of the CSFV strains. Multiple sequence alignments were performed using ClustalX, and phylogenetic trees were constructed from the aligned nucleotide sequences using the neighbor-joining method. Bootstrap values greater than 50% based on 1000 replicates are shown at each branch. The names of the strains, countries and dates (year) of isolation, GenBank accession numbers, and genotypes with subgroups are shown. Solid circles indicate the 2017–2018 strains identified in this study; solid squares indicate the recent strains identified on Jeju Island in 2016; solid diamonds indicate the reference LOM and commercial vaccine strain. Scale bars indicate the nucleotide substitutions per site.

Since the LOM vaccine was accidently supplied by a vaccine-manufacturing company and used on non-vaccinated Jeju pig herds in 2014, there has resulted in substantial damages to the pig farms on the island. Furthermore, the virus has persistently circulated within the infected herd, engendering recurrent outbreaks (Choe et al., 2019). Considering accidental vaccination occurred only in 2014, LOM-derived field isolates have spread province-wide via farm-to-farm transmission, suggestive of an endemic situation on Jeju Island. The early outbreaks affected pregnant sows with typical reproductive problems including stillbirth and fetus mummification, while clinical manifestations of the recent CSF mainly included severe atrophy and diarrhea accompanied by high mortality in pre- and post-weaning pigs. Given the latter clinical characteristics under current field circumstances, I surmise that at the time of transplacental infection, the virus enters the fetus and is recognized as a self during immune system development (immunological tolerance), leading to the birth of persistently infected (PI) offspring. Such piglets may initially appear to be clinically normal, but subsequently begin to develop the gastrointestinal infection causing diarrhea, which is invariably fatal and age-dependent. The PI animals that do survive may continuously shed the virus and become important reservoirs of CSFV for horizontal transmission. Further studies are required for investigating the biological and pathogenic characteristics of the LOM-derived field isolates in natural hosts.

Sequence comparison and phylogenetic analyses indicated that the Jeju LOM-derived field isolates were most genetically similar to an original LOM strain or a commercial vaccine, but have experienced lasting genetic drift under field circumstance. A previous study showed that LOM

vaccination in naïve pregnant sows could induce fetal abnormalities, suggesting its inherent safety concerns including transplacental transmission and fetal death (Lim et al., 2016). The whole genomic sequence of the LOM strain KNU-1823 identified from the aborted fetus at 50–60 days of pregnancy was nearly identical to that of a reference LOM and a commercial vaccine strain, showing 5-aa/1-nt (3′-UTR) and 2-aa differences, respectively. This result further supported the reality of vaccine adverse effects in pregnancy and fetuses. In contrast, comparative analysis of paired LOM/LOM-derived field strains showed 111-nt substitutions leading to 28 non-silent mutations throughout the entire genome in other aborted fetus at 1–2 weeks prior to farrowing (KNU-1824) and 127–148-nt substitutions leading to 31–42 non-silent mutations in neonates and young pigs with CSFV-associated mortality (KNU-1825, -1826, and -1827). Although it remains unknown whether it is the independent aa mutations or multiple mutations combined that lead to the acquisition of its innate low pathogenicity of the LOM vaccine strain, more than two-thirds of the substitutions were accumulated in the non-structural protein (NSP) coding regions, particularly NS4B, NS5A, and NS5B. These non-structural proteins may play roles in virus evasion of host defense mechanisms as innate immune antagonists and accordingly, provide opportunities for the virus to revert to its original virulence. Thus, cutting-edge research using reverse genetics will be necessary to provide fundamental insights into the specific role of NSP mutations in CSFV pathogenesis.

Since the initial introduction of the LOM vaccine, genetic changes possibly associated with the subsequent reversion to virulence have continually occurred in the virus under selective pressures via multiple

environmental factors on Jeju Island. These include densely populated pig farms, indiscreet feedback practices to control porcine epidemic diarrhea since its re-emergence in 2014 (Lee et al., 2014), and scant information and lax biosecurity regarding LOM transmission under CSFV naïve situations. The resulting occurrence of evolutionary processes necessary for viral fitness appears to recover virulence and eventually develop LOM-associated clinical diseases and sequential transmission of the pathogen. More importantly, the LOM-derived field strains still contain more than 100 silent mutations due to genetic drift. As the virus continues to evolve, those substitutions may spontaneously shift to explosive non-silent aa changes, warranting concerns about unexpected and uncontrolled CSFV outbreaks. Therefore, I need to operate a monitoring and surveillance system to trace the mutation states of the LOM strain circulating in the field, re-evaluate the use of the LOM vaccine by reviewing its safety characteristics, and spur the development of new vaccines guaranteed to be safe. In conclusion, I urgently require the gathering of genetic and biological data of the current LOM-derived field strains to establish strict biosecurity policy and simultaneously apply control measures to control and eradicate CSF. These actions include actively removing PI animals and blocking inter- and intra-farm fecal transmission in addition to the use of alternate vaccines not only to provide stable herd immunity, but also to permit the differentiation of infected from vaccinated animals. Together, this will help halt the current widespread and continuous devastation caused by LOM on Jeju Island. The present study will provide insights into better understanding of the genetic alterations of the attenuated live LOM vaccine under environmental pressures that may facilitate its potential reversion.

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Chapter 2

Identification and molecular characteristics of classical swine fever virus LOM variants with 3′-UTR INDELs

1. Abstract

The classical swine fever virus (CSFV) reemerged in naïve pig herds on Jeju Island, South Korea, due to the accidental introduction of the LOM vaccine strain in 2014. Since this reemergence, the free region has experienced numerous outbreaks, leading to the appearance of endemics in provincial herds. In this study, I investigated the complete genome sequences and the molecular characterization of LOM-derived field CSFV strains with unique insertion-deletion (INDEL) mutations in the 3′-untranslated region (UTR), responsible for ongoing sporadic outbreaks on Jeju Island in 2019. The 2019 emergent Jeju LOM-derived variants had their own INDEL signatures in the 3′-UTR, resulting in changes to the predicted secondary stem-loop structures. The genomes of these strains were 12,297–12,302 nucleotides in length, one-nucleotide (nt) shorter or one-, two-, or four-nt longer than the reference LOM strain. The 3′-UTR INDEL variants shared 98.8–99.0% and 98.3–98.6% identity with the LOM strain at the polyprotein and full-genome levels, respectively. The total number of genetic variations between the LOM vaccine strain and the 3′-UTR INDEL isolates ranged from 161–202 and 37–45 at the nucleotide and amino acid levels, respectively. These mutations were broadly dispersed throughout the genome and particularly clustered in NS2 and 3′-UTR, possibly triggering a reversion to low virulence and allowing the virus to adapt to improve its persistence in the field. This study provides important information about the genetic evolution of the LOM-derived CSFV circulating in the free region, which arises from continuous non-lethal mutations to ensure viral fitness in host animals.

Key words: CSFV; Endemic outbreaks; Jeju Island; LOM-derived field strains; Reversion

2. Introduction

Classical swine fever (CSF) is one of the most serious transboundary viral diseases affecting domestic pigs and wild boar (Blome et al., 2017). Because of its tremendous economic impact on the global pig industry, CSF is notifiable to the World Organization for Animal Health (OIE) (Moennig et al., 2003). The disease is caused by the CSF virus (CSFV), a small enveloped single-stranded, (+) sense RNA virus of the genus *Pestivirus* included into the *Flaviviridae* family; recently, it was taxonomically renamed *Pestivirus C* (Smith et al., 2017). Although CSF has been successfully eradicated in several countries, it remains sporadic or endemic in most parts of the world with industrialized pig production, including Asia, Eastern Europe, and South and Central America (Blome et al., 2017). For effective CSF control, live attenuated vaccines are used to reduce the disease burden in most endemically infected countries (Ji et al., 2015). Similarly, South Korea has implemented a nationwide vaccination policy using an attenuated LOM strain that derives from a low-virulence strain of a Japanese Miyagi isolate to combat CSF over the past four decades (Kim et al., 2008). Despite this national vaccination campaign, only Jeju Province, the largest island of South Korea located 80 km away from the mainland at its closest point, successfully became a CSF-free region in 1999 and had then maintained the free status for over 10 years (Song et al., 2013). However, the CSFV reemerged on Jeju Island in 2014 following the unintentional infection of the immunologically naïve pig population with the LOM strain (Jang et al., 2019; Je et al., 2018). The LOM vaccine strain has since spread through the provincial stock farms and has now

become endemic in the western region of Jeju Island, undergoing continuous genetic mutations (Jang et al., 2019).

3. Materials and Methods

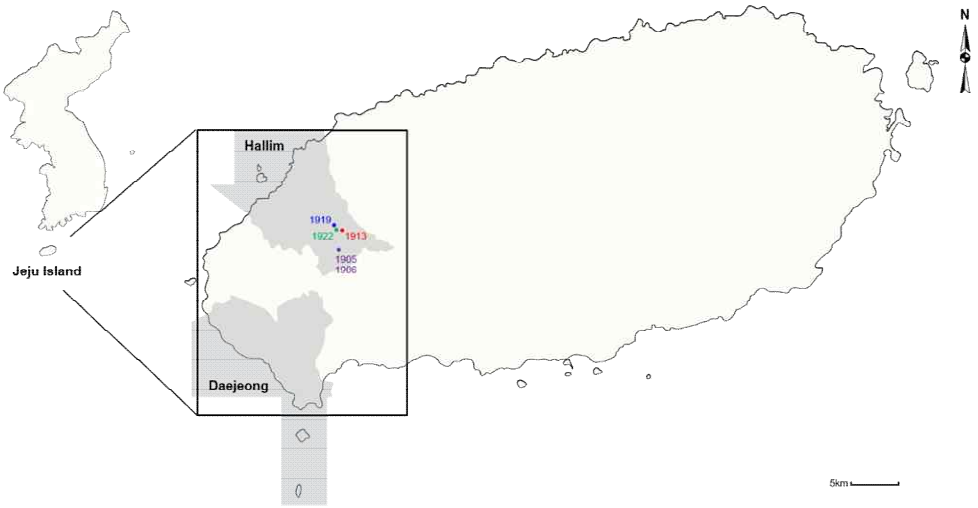
3.1. Clinical sample collection

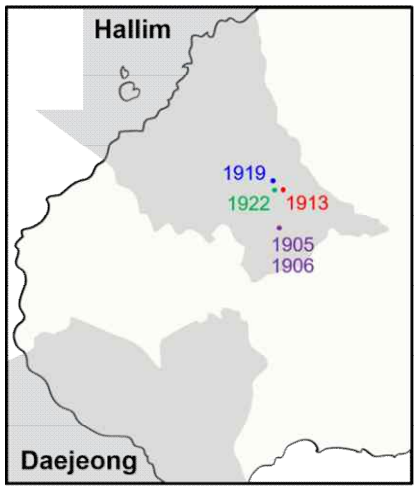
From January to July 2019, clinical specimens of CSF-suspected suckling or fattening pigs that huddled together with dermatitis and diarrhea or that experienced severe atrophy before dying were independently submitted by four different unvaccinated swine farms for laboratory analysis (Table 1). These specimens were prepared as described previously (Jang et al., 2019) and tested LOM positive following the combined RT-PCR and sequencing analysis (Kim et al., 2008).

Table 1 2019 CSF cases from which the LOM-derived strains were identified in this study

	Incidence type (date)	Ages of affected pigs	Clinical manifestation	Mortality rate[a]	Sample type
Farm A (KNU-1905)	Recurrence[b] (January)	Fattening pigs (90–120 days)	Dermatitis, fever, and diarrhea	10–15%	Lymph node
Farm A (KNU-1906)	Recurrence (March)	Fattening pigs (90–120 days)	Dermatitis, fever, and diarrhea	10–15%	Blood
Farm B (KNU-1913)	Recurrence[c] (April)	Suckling piglets (1–5 days)	Atrophy, fever, diarrhea, dermatitis, and death	20–30%	Spleen, kidney, lung, and liver[f]
Farm C (KNU-1919)	Recurrence[d] (June)	Suckling piglets (1–5 days)	Atrophy, diarrhea, and death	50% (3 litters)	Spleen, kidney, lung, and liver[f]
Farm D (KNU-1922)	Rccurrence[e] (July)	Suckling piglets (1–5 days)	Atrophy, congenital tremor, & death (stillbirth)	33% (1 litter)	Spleen, kidney, lung, and liver[f]

[a]The rate in the corresponding age group or litters
[b]The first LOM confirmed in 2015
[c]The first LOM confirmed in 2016
[d]The first LOM confirmed in 2016
[e]The first LOM confirmed in 2016
[f]Pooled for virus detection





3.2. Nucleotide sequence analysis

The full-length genomic sequences of the 2019 Jeju LOM isolates were determined using the traditional Sanger sequencing method, as described previously (Jang et al., 2019; Lee and Lee, 2013). The sequence data were deposited in GenBank under the accession numbers MN399380 through MN399384.

3.3. Multiple alignments and phylogenetic analyses

The complete sequences of the 55 E2 genes and 51 genomes of global CSFV strains were independently used in sequence alignment and phylogenetic analyses. Multiple sequence alignments were generated with the ClustalX 2.0 program (Thompson et al., 1997) and the percentages of nucleotide sequence divergences were further assessed using the same software program. Phylogenetic trees were constructed from the aligned nucleotide or amino acid sequences by using the neighbor-joining method and subsequently subjected to bootstrap analysis with 1000 replicates to determine the percentage reliability values of each internal node of the tree (Saitou and Nei, 1987). All Figures involving phylogenetic trees were generated using Mega 4.0 software (Tamura et al., 2007).

3.4. secondary structure prediction

The effect of the 3′-UTR INDEL mutations on the predicted 3′-UTR RNA secondary structure was compared for the LOM vaccine and the 2019 field isolates using the mfold Web Server software (The RNA Institute College of Arts and Science University at Albany, New York).

4. Results and discussion

Genetic analysis revealed high homology among the 2019 Jeju LOM isolates, designated as KNU-1905, -1906, -1913, -1919, and -1922, ranging from 94.2 to 99.7% nucleotide (nt) and 98.3 to 99.9% amino acid (aa) similarity at the genomic level. The 2019 LOM isolates also shared a high identity with the LOM vaccine strains and 2017–2018 Jeju isolates, with 94.3 to 99.5% nt and 98.3 to 99.7% aa homology, respectively. Compared to the LOM vaccine, the total number of nt/aa differences in the polyprotein-coding regions of the 2019 isolates ranged from 161/37 to 202/45, with the aa variations observed extensively in four structural (C, E^{rns}, E1, and E2) and seven nonstructural (N^{pro}, p7, NS2, NS3, NS4B, NS5A, and NS5B) proteins (Figure 1A). The locations of the genomic mutations in the most recent isolates, KNU-1913, -1919, and -1922, were similar to each other and comparable to those in the 2017–2018 strains. However, the genetic drift positions (i.e., barcode patterns) for KNU-1905 and -1906 were clearly distinguishable from those for other 2017–2018 isolates due to the notable increase in the total number of aa mutations within the genome to 44–45, particularly in relation to NS2. Although the 5′-untranslated region (UTR) has remained unchanged or has experienced variations of only 2–5-nt since the 2014 re-emergence, the 3′-UTR of the 2019 isolates represent a mutation hotspot, exhibiting the 8–12-nt substitutions, three times higher than observed in the 2017–2018 isolates (1- or 3-nt) (Figure 1A). The similarity in the nt/aa identities and the number of nt/aa differences between the 2019 Jeju isolates and the reference LOM vaccine strain are summarized in Table 2. As shown in Figure 1B, genetic

changes in the CSFV LOM strain have persistently arisen across the whole genome since its introduction in 2014. In the protein-coding regions, the C, E^{rns}, P7, NS2, and NS4B proteins showed a divergence of > 2% between the contemporary LOM isolates and the reference strain (Figure 1B and Table 2). Furthermore, the 3′-UTR underwent nearly up to 5% divergence in 2019, showing the greatest dissimilarity. The evolutionary rates of nt substitutions/site/year ($\times 10^{-3}$) in CSFV LOM strains were estimated to be 2.2802 and 1.2402 for the E2 gene and full-length genome, respectively (Drummond et al., 2012). Subsequent E2 gene- or whole-genome-based phylogenetic analysis clearly divided the CSFV into three genotypes (1, 2, and 3), each of which was subdivided into representative sub-genotypes (Figure 2). All of the Jeju LOM-derived field strains identified in 2019 were grouped within the sub-genotype 1.1 clade, which clustered together with the LOM vaccine strains and past Jeju isolates; however, the evolutional distance between the LOM vaccine strains and the 2019 LOM variants increased, and in particular, KNU-1905 and -1906 formed an independent branch within the LOM-related clade. Taken together, the genetic and phylogenetic data indicate that there has been continuous genetic evolution of the LOM-derived CSFV circulating in the free field.

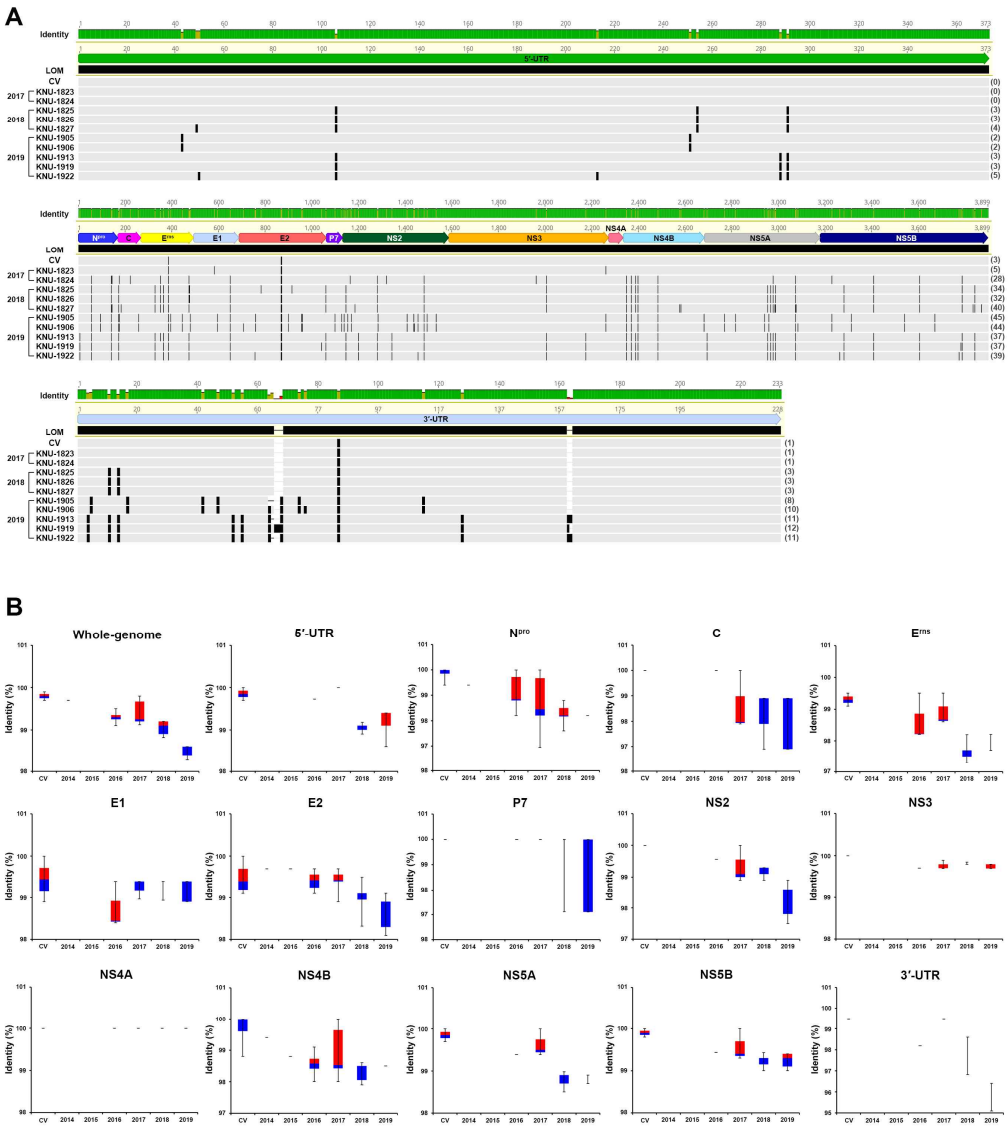


Figure 1. Genetic variations in the CSFV LOM-derived field isolates identified during the reemergence on Jeju Island. (A) Schematics of nucleotide (nt) and amino acid (aa) differences between the reference LOM vaccine strain and the 2017–2019 isolates. The top diagram in each panel composing the CSFV genome represents the 5′-UTR, a single polyprotein (N^{pro}, C, E^{rns}, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B), and the 3′-UTR, respectively. The vertical black bars represent a 1-nt or

1-aa mutation in relation to the nt or aa sequence of the reference LOM strain. The thin horizontal dashed lines (bottom panel) indicate deleted or inserted nucleotides in the 3′-UTR. The numbers within the parenthesis on the right indicate the number of individual differences in the 5′-UTR, polyprotein, and 3′-UTR, respectively, compared to the LOM strain. (B) The box plots show the nt or aa identity percentage for the CSFV LOM-derived field isolates compared to the LOM vaccine at the genomic or each gene level by year. The boundary of the blue boxes to zero indicates the 25^{th} percentile, the line within the boxes marks the median, and the boundary of the red box farthest from zero indicates the 75^{th} percentile. The error bars above and below the boxes indicate the maximum and minimum scores for each data set. CV denotes commercial LOM vaccines in the market.

Table 2 Comparison of nucleotide (nt) and amino acid (aa) sequence identities of LOM-derived field isolates with LOM vaccine strain

Strain	Nucleotide/amino acid identity (%) (No. of nucleotide/amino acid differences)														
	5′ UTR	N^{pro}	C	E^{ms}	E1	E2	P7	NS2	NS3	NS4A	NS4B	NS5A	NS5B	3′ UTR	Total
KNU-1905	99.4 (2)	97.8/98.2 (11/3)	98.3/96.9 (5/3)	98/98.2 (13/4)	98.6/98.9 (8/2)	98.3/98.3 (18/6)	98/97.1 (4/2)	98/97.8 (26/10)	98.4/99.8 (31/1)	98.9/100 (2/0)	97.7/98.5 (23/5)	98.8/98.9 (17/5)	98.7/99.4 (27/4)	95.6 (8)	98.4/98.8 (195/45)
KNU-1906	99.4 (2)	97.8/98.2 (11/3)	98.3/96.9 (5/3)	98/98.2 (13/4)	98.6/98.9 (8/2)	98.3/98.1 (19/7)	98/97.1 (4/2)	98/97.5 (27/11)	98.4/99.8 (32/1)	98.9/100 (2/0)	97.7/98.5 (23/5)	98.6/98.7 (20/6)	98.7/99.4 (26/4)	96.4 (10)	98.3/98.8 (202/44)
KNU-1913	99.1 (3)	98.2/98.2 (9/3)	98.3/98.9 (5/1)	97.9/97.7 (14/5)	98.9/99.4 (6/1)	99.1/99.1 (10/3)	96.6/100 (7/0)	98.9/98.6 (15/6)	99.1/99.7 (17/2)	100/100 (0/0)	98.2/98.5 (18/5)	98.1/98.7 (27/6)	99.1/99.3 (19/5)	95.6 (11)	98.6/99 (161/37)
KNU-1919	99.1 (3)	98.2/98.2 (9/3)	98.3/98.9 (5/1)	97.6/98.2 (16/4)	98.8/99.4 (7/1)	99.0/98.9 (11/4)	96.6/100 (7/0)	98.9/98.9 (14/5)	99.2/99.7 (16/2)	100/100 (0/0)	98.2/98.5 (18/5)	98.3/98.7 (25/6)	98.9/99.1 (22/6)	95.1 (12)	98.6/99 (165/37)
KNU-1922	98.6 (5)	98.2/98.2 (9/3)	98.3/98.9 (5/1)	97.7/98.2 (15/4)	98.8/99.4 (7/1)	99.1/98.9 (10/4)	96.6/100 (7/0)	98.9/98.6 (14/6)	99.2/99.7 (15/2)	100/100 (0/0)	98.2/98.5 (18/5)	98.4/98.7 (23/6)	98.9/99 (22/7)	95.6 (11)	98.6/98.9 (161/39)

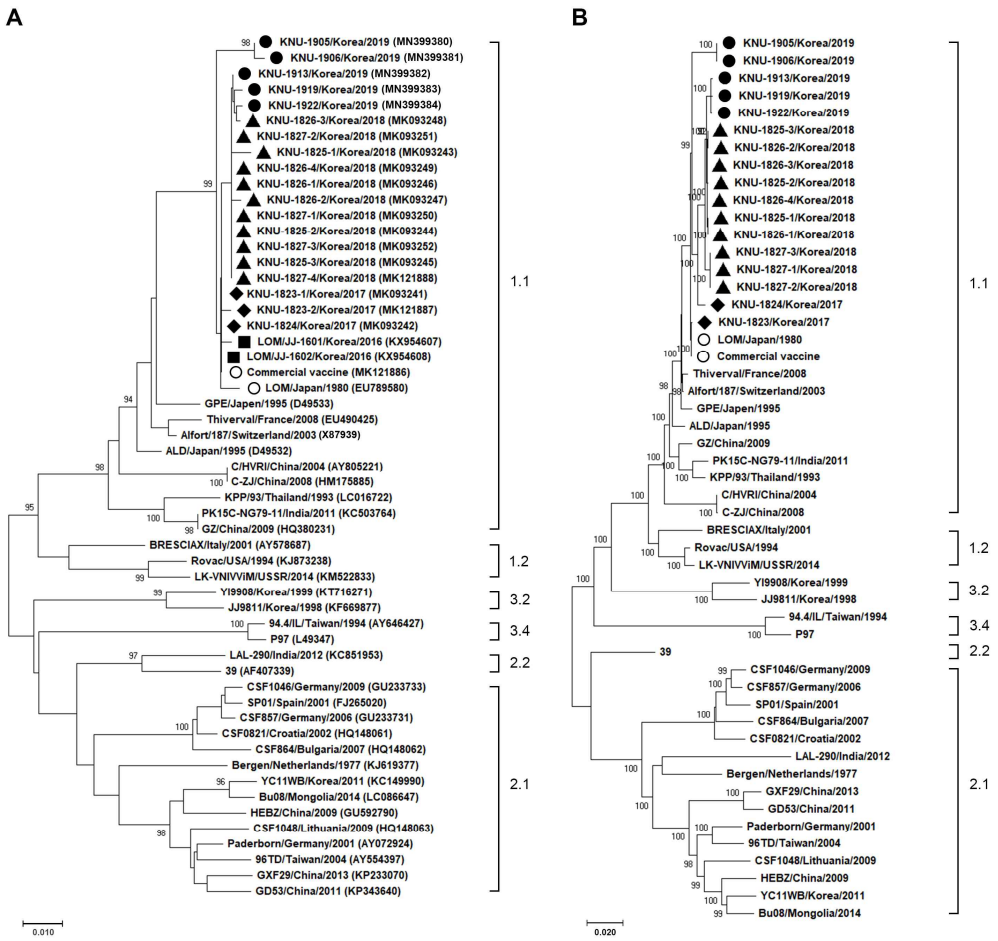


Figure 2. Phylogenetic analyses based on the mucleotide sequences of the E2 genes (A) and full-length genomes of the CSFV strains. Multiple sequence alignments were performed using ClustalX, and phylogenetic trees were constructed from the aligned nucleotide sequences using the neighbor-joining method. Bootstrap values greater than 50% based on 1000 replicates are shown at each branch. The names of thestrains, countries and dates (year) of isolation, GenBank accession numbers, and genotypes with subgroups are shown. Solid circles indicate the 2019 strains identified in this study; solid triangles indicate the Jeju strains identified in 2018; solid diamonds indicate the Jeju strains identified in 2017; solid squares indicate the Jeju strains identified in 2016; open circles indicate the reference LOM and commercial vaccine strain. Scale bars indicate nucleotide substitutions per site.

Interestingly, all of the isolates independently harbored novel deletion or insertion (INDEL) mutations in the 3′-UTR (3′-UTR INDELs) that have been unidentified in the global CSFV strains available in the GenBank database; these include a uridine (U) deletion at the genomic position 12,135 in KNU-1905, an adenine (A) insertion between the genomic positions 12,135 and 12,136 in KNU-1906, UU insertions between the genomic positions 12,229 and 12,230 in KNU-1913 and -1922, and UUA and U discontinuous insertions between the genomic positions 12,135 and 12,136 and 12,229 and 12,230 in KNU-1919 (Figure 3A). No additional INDELs were identified in the entire genomes of the 2019 field strains. Due to these INDELs, the length of the 3′-UTR sequences differed among the variants, with sizes of 227-nt for KNU-1905, 229-nt for KNU-1906, 230-nt for KNU-1913 and -1922, and 232-nt for KNU-1919, making them one-nt shorter and one-, two-, and four-nt longer, respectively, than the LOM vaccine strain, leading to a divergent genome of 12,297–12,302 nucleotides. A secondary structure consisting of four stem-loops (SL-I to SL-IV) was obtained from the 3′-UTR of LOM (Figure 3B) (Fan et al., 2008). However, the INDEL mutations in the 2019 LOM variants led to major changes in the structures of the SL-II and/or SL-III and in the distances of the single-stranded intervening sequences (SS) between the SL-I and SL-II and the SL-II and SL-III when compared to the LOM vaccine. In addition, the nucleotide substitutions accumulated around the 5′-terminal part of the 3′-UTR at the genomic positions 12,122–12,154 altered the SL-III structure, creating two sub-SLs, SL-III.1 and SL-III.2. Further virus isolation and sequencing studies showed that the LOM viruses isolated from each clinical samples maintained the 3′-UTR INDEL mutations without additional genetic drift, which were stable during the subsequent serial passages (Figure 4).

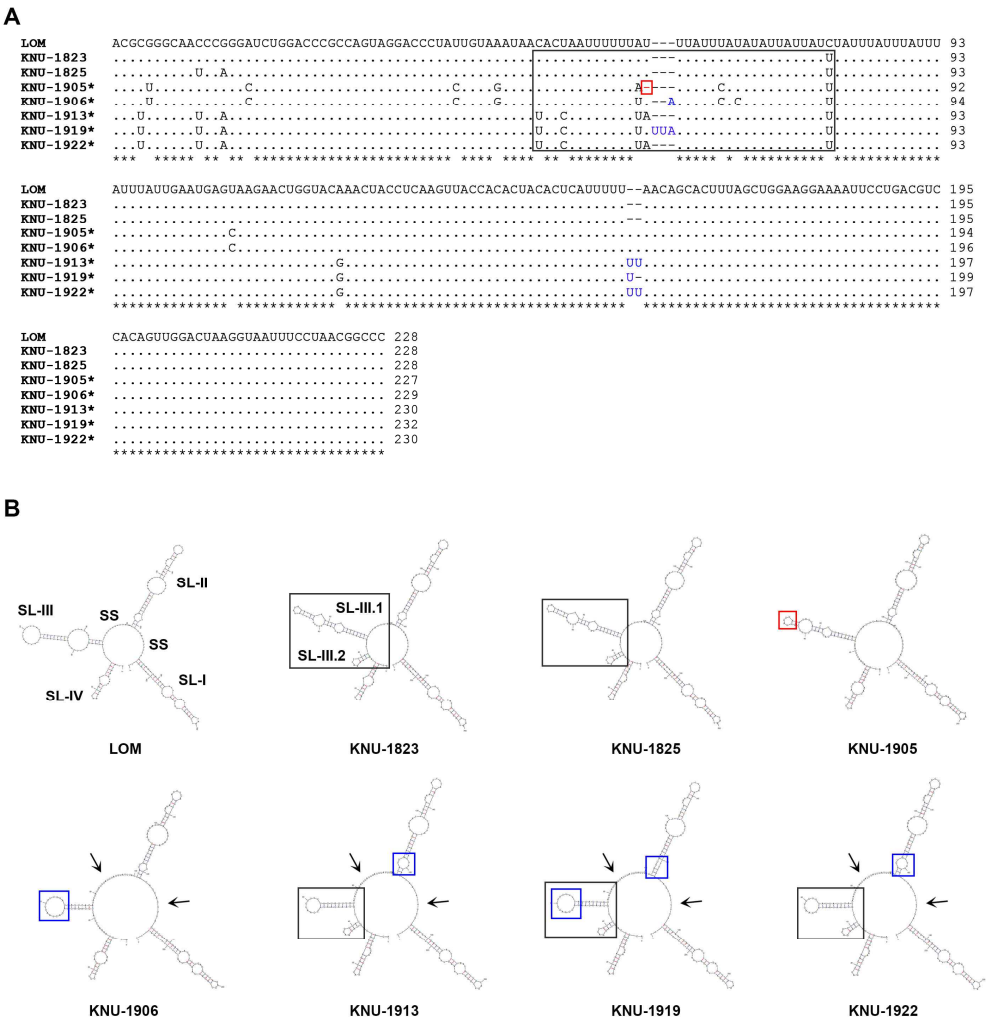


Figure 3. Alignment and secondary structure prediction of the 3′-UTR of the LOM vaccine strain and the LOM-derived field isolates. (A) The 3′-UTR INDEL variants KNU-1905, -1906, -1913, -1919, and -1922 identified in this study are marked with an asterisk. A red dash in a red box and the use of blue indicate a deleted sequence and an inserted nucleotide, respectively. Nucleotide substitutions affecting the stem-loop (SL) structure of the 3′-UTR are also indicated in a box. (B) The basic profile is composed of four SL structures, SL-I, -II, -III, and IV, and region SS

as the anticlockwise orientation. The boxes indicated major changes in the 3′-UTR SL-II or/and SL-III structures resulting from a deletion (red), insertions (blue), or substitutions (black). The arrows indicate longer SS regions in the 3′-UTR of the LOM isolates. LOM (reference strain), free energy = −48.80; KNU-1823, free energy = −48.00; KNU-1825, free energy = −50.40; KNU-1905, free energy = –45.90; KNU-1906, free energy = −45.50; KNU-1913, free energy = −50.40; KNU-1919, free energy = −50.80; KNU-1922, free energy = −50.40.

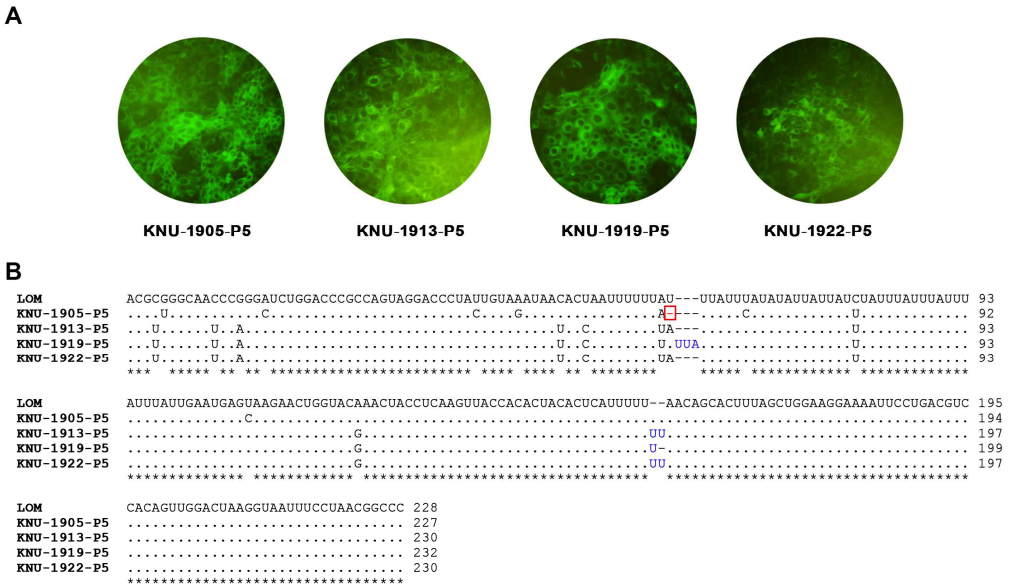


Figure 4. Virus isolation of the LOM-derived field isolates and the 3'-UTR alignment of the passage 5 (P5) viruses. (A) CSFV isolation was conducted from filtered clinical samples on LLC-PKI cells. Briefly, Confluent LLC-PKI cells grown in 6-well plates were washed with PBS anc inoculated with 300 μl of each inoculum. After incubating at 37℃ for 1 h to allow for viral adsorption, 2 ml of cell growth medium was added to each well. The inoculated cells were maintained at 37℃ under 5% CO_2 for five days. The infected cells were subjected to three rounds of freezing and thawing. The clarified supernatants were aliquoted and stored at –80℃ as passage 1 (P1) viral stocks for use in subsequent serial passages. Virus propagation was confirmed by the detection of CSFV antigens with immunofluorescence assay using a CSFV E2-specific antibody (CAVAC, Daejeon, South Korea). (B) The 3′-UTRs of the indivisual P5 viruses were sequenced as described previously. Nucleotide sequence alignment of the CSFV 3′-UTR was generated using the ClustalX 2.0 program (bottom panel). A red dash in a red box and the use of blue indicate a deleted sequence and an inserted nucleotide, respectively.

Since the LOM vaccine was inadvertently released onto previously CSF-free Jeju Island in 2014, the vaccine strain has continued to circulate within and between the farms without any reduction in prevalence, raising safety and revaluation concerns about the national sole vaccine, posing a health and economic threat to pigs and farmers, and leading to the evolution and reversion of the virus (Jang et al., 2019; Je et al., 2018). The present study reports the molecular characterization of novel LOM variants with INDELs in the 3′-UTR that are currently found in the Hallim area on Jeju Island. Sequence comparison and phylogenetic analysis confirmed persistent genetic changes of the LOM isolates in the face of evolutional pressures and identified hotspots, including C, E^{rns}, P7, NS2, NS4B, and 3′-UTR, where genetic mutations have been significantly elevated in 2019. In particular, the 3′-UTR of the 2019 LOM-derived virus contained strain-specific INDEL mutations that, along with nucleotide substitutions, have resulted in modifications to consecutive stem-loop structures and increases in the SS distances within the LOM 3′-UTR. The pestivirus 3′-UTR harbors several *cis*-acting elements essential for viral replication and translation control, with SL-I and the SS region between SL-I and SL-II particularly vital for pestivial replication (Huang et al., 2012; Pankraz et al., 2005). The 3′-UTR in the CSFV is dispensable for viral replication *in vitro* and *in vivo* but is indispensable for viral pathogenesis (Li et al., 2014). It is noteworthy that large U-rich insertions (6- to 32-nt) have been found in the 3′-UTR of several attenuated CSFV vaccine strains, indicating the acquisition of the U-rich sequences during the viral adaptation (Fan et al., 2008; Wu et al., 2001). Furthermore, a poly-U tract with an average 36-nt length insertion was found in the 3′-UTR of a

low virulent CSFV strain isolated after persistence in newborn piglets (Coronado et al., 2017). Given its critical functions in viral replication and pathogenesis, the INDEL and substitution mutations in the 3′-UTR of the LOM isolates might be associated with a reversion to its original low pathogenic virulence and a resulting potential advantage in terms of adapting to the endemic circumstances and facilitating the establishment of persistent and chronic infections. In addition, the possibility of the involvement of genetic drift at other hotspots in such viral fitness cannot be excluded and remains to be determined. Therefore, future research that employs animal experiments and reverse genetics is needed to assess the pathogenicity of the LOM-derived variants and to investigate the implications of genetic mutations in hotspots, including the 3′-UTR in viral evolution. Currently, most cases of CSFV detection in herds on Jeju Island have been confirmed on farms with recurrent LOM infections, between which the clinical manifestations, the ages, the number of affected pigs or litters, and the mortality rates for the affected age group or litter varies (Table 1). Under these conditions, it will be important to identify subclinical or asymptomatic animals that have been infected but survived within an affected age group or litter. They may eventually become persistently infected (PI) pigs (i.e., carriers and shedders) that can transiently or continuously shed the virus and act as the phantom menace to accelerate horizontal transmission among pigs or farms. Thus, the removal of PI survivors from affected litters is one of the imperative tasks to break the chain of recurrence or preventing new occurrences. In conclusion, the LOM virus continues to genetically evolve under recurrent or endemic pressure on hitherto unvaccinated Jeju Island, previously free of

CSF, possibly favoring reversion and persistence in the field. To counteract the attentions and risks highlighted in this study, which may worsen the current situation in the province and promote the possibility of uncontrolled endemics, appropriate control measures, including biosecurity practices, vaccination (using alternative vaccines), monitoring, and surveillance, need to be implemented.

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Chapter 3

Pathogenic characteristics of novel classical swine fever virus LOM 3′-UTR INDEL variant

1. Abstract

Reemergent local outbreaks of classical swine fever (CSF) occurred simultaneously in multiple pig farms on CSF-free Jeju Island, South Korea, in 2014 because of inadvertent injection of a commercial CSF (LOM) vaccine into pregnant sows. The LOM virus has since spread across the island and has become endemic in Jeju herds, raising concern about possible reversion to virulence of the LOM vaccine. I previously isolated LOM-derived field CSF virus (CSFV) strains with unique insertion-deletion (INDEL) mutations in the 3′-untranslated region (UTR), designated LOM-derived Jeju 3′-UTR INDEL variants, from CSF-recurrent swine farms on Jeju Island in 2019. The present study conducted animal experiments to investigate whether a 2019 emergent LOM 3′-UTR INDEL variant, KNU-1905, has reverted to a pathogenic form in conventional pigs. Experimental animal infection showed that pigs inoculated with the commercial LOM vaccine strain developed no adverse effects compared to the sham-infected pigs. However, KNU-1905 displayed pathogenic characteristics in pigs, including clinical symptoms (e.g., lethargy, conjunctivitis, nasal discharge, and diarrhea), weight loss, and gross lesions. Moreover, viremia, virus shedding in feces and nasal fluids, and viral loads in various tissues of all the KNU-1905-infected pigs were highly significant, in contrast to those of the LOM-infected group in which CSFV RNA was detected only in the serum, nasal, and tonsil samples of one identical pig. In conclusion, the LOM-derived field isolate with molecular variations induced clinical adverse events in pigs, which commonly shed considerable amounts of CSFV. This study provides evidence that the

genetic evolution of the LOM-derived CSFV circulating on Jeju Island might have allowed the LOM vaccine to recover its primary prototype and that these variants might have induced chronic or persistent infection in pigs that can shed CSFV in field farms leading to a risk of transmission among pigs or farms in this former CSF-free region.

Key words: CSFV, Jeju Island, LOM-derived variant, MLV-LOM, pathogenicity, reversion-to-virulence

2. Introduction

Classical swine fever (CSF) is a highly contagious multisystemic viral disease of domestic pigs and wild boars with an enormous socio-economic impact on animal health and production (Blome et al., 2017). The CSF virus (CSFV), the causative agent of the disease, is a small, enveloped, single-stranded, (+) sense RNA virus of the genus *Pestivirus* in the family *Flaviviridae*; recently, this species was re-designated as *Pestivirus C* (Smith et al., 2017). The CSFV genome is approximately 12.3-kb long and contains one large open reading frame (ORF) flanked by two untranslated regions (UTRs) at both ends, the uncapped 5′-UTR with an internal ribosome entry site and the uridine-rich 3′-UTR. The single ORF encodes one precursor polyprotein that undergoes co- and post-translational processing by viral and cellular proteases to produce 12 mature proteins: four structural (C, E^{rns}, E1, and E2) and eight non-structural proteins (N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (Blome et al., 2017; Ganges et al., 2020; Ji et al., 2015; Tautz et al., 2015).

CSF can be divided into acute (lethal), chronic, and persistent forms of the disease. The acute pattern is characterized by high fever, gastrointestinal symptoms, neurological signs, skin hemorrhages or cyanosis, and high mortality depending on the virulence of the virus strain and the age of the animal (Blome et al., 2017; Moennig et al., 2003; Petrov et al., 2014). Chronic CSF occurs in infected pigs that fail to mount an effective immune response to clear the virus from circulation. Although the outcome

of the chronic disease is always fatal, affected animals can live for months and constantly shed large amounts of CSFV (Blome et al., 2017). The persistent course of CSF usually occurs when the virus infects pregnant sows, leading to vertical transmission to the fetus. Unlike the infected sows, which show mild clinical signs, CSFV infection results in fetal mummification, abortions, or stillbirth, depending on the strain and the gestation time. However, infection at 50–70 days of gestation can induce an immunotolerance phenomenon, leading to the birth of persistently infected (PI) offspring. The PI piglets appear to clinically normal but die due to the so-called late-onset CSF after several months. During this period, they can shed viral loads sufficient for transmission and thus together with chronically infected pigs, act as virus reservoirs (Bohórquez et al., 2020; Frey et al., 1980; Hermanns et al., 1981; Kaden et al., 2005; Meyer et al., 1981; Richter-Reichhelm et al., 1980; Stewart et al., 1973; Vannier et al., 1981; von Benten et al., 1980).

Strict intervention strategies, including quarantine and stamping out of affected herds with or without vaccination, allowed several countries to gain CSF-free status. Nevertheless, CSF remains endemic in South and Central America, Eastern Europe, and Asia (Blome et al., 2017; Ganges et al., 2020). Highly efficacious live-attenuated vaccines have existed for many decades and paved the road to CSF eradications (van Oirschot, 2003). These vaccines have been compulsorily implemented for effective CSF control in endemic regions (Ji et al., 2015), and in 2019, 25 countries officially enforced mandatory vaccination campaigns (OIE WAHIS, 2021). Likewise, South Korea has maintained a nationwide mandatory immunization policy using a CSF-modified live vaccine (MLV) based on an

attenuated form of a low-virulence strain of the Miyagi isolate (LOM) from Japan (MLV-LOM) (Kim et al., 2008). Owing to its CSF-free status, Jeju Province, the largest island of South Korea, where vaccination was banned in 1998, has been exempted from this mandate (Song et al., 2013). However, since the provincial no-vaccination policy, Jeju Island has experienced five outbreaks because of the unintentional introduction of the MLV-LOM into CSFV-naïve pigs via unexpected routes from mainland South Korea (Choe et al., 2019; Kim et al., 2008).

The most recent reemergence of CSFV on Jeju Island began in 2014 by the accidental inoculation of naïve sows with a commercial CSF LOM-swine erysipelas combined live vaccine (Jang et al., 2019; Je et al., 2018;). Although vaccination was halted immediately, the LOM vaccine strain has spread via farm-to-farm transmission and has affected more than 100 pig farms. At present, the LOM-derived CSFV strain is endemic in the western region of Jeju Island and undergoing substantial genetic drift (Jang et al., 2019). Furthermore, our previous study reported LOM-derived field CSFV variants with unique insertion-deletion (INDEL) mutations in the 3′-UTR responsible for current sporadic outbreaks on Jeju Island (Jang et al., 2020). Due to suspicion regarding the safety and reversion-to-virulence of the commercial LOM vaccine strain, this study aimed to investigate pathogenic traits of a LOM Jeju variant with 3′-UTR INDEL *in vivo*.

3. Materials and Methods

3.1. Cells and viruses

LLC-PK1 cells (ATCC CL-101) were cultured in alpha-minimum essential medium (α-MEM; Invitrogen, Carlsbad, CA) with 5% fetal bovine serum (FBS; Invitrogen) and penicillin-streptomycin (100×; Invitrogen). The cells were maintained at 37°C in an atmosphere of humidified air containing 5% CO_2. The commercial CSFV MLV-LOM strain (GenBank accession number: MK121886) was obtained from ChoongAng Vaccine Laboratories (CAVAC; Daejeon, South Korea) (Jang et al., 2019). The LOM-derived Jeju strain KNU-1905 (GenBank accession number: MN399380) was isolated and maintained in our laboratory (Jang et al., 2020). The viruses were independently propagated in LLC-PK1 cells, as described previously (Jang et al., 2020). Individual viral stocks were prepared from each fifth-passage cell culture (LOM-P5 and KNU-1905-P5) and used as the challenge virus in this study.

3.2. Pig infection experiments

Ten 4-week-old crossbred pigs (Great Yorkshire × Dutch Landrace) were obtained from a commercial farrow-to-finish farm with good health status and no previous CSF outbreak or vaccination history in Jeju Province and were tested to confirm that they were not infected with CSFV. All animals were also determined to be negative for porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus 2 (PCV2) by virus-specific RT-PCR, as described previously (Jang et al.,

2021; Park et al., 2020). Pigs were randomly assigned to three experimental groups: LOM-derived Jeju isolate KNU-1905-inoculated group 1 (n = 4), LOM vaccine-inoculated group 2 (n = 4), and sham-inoculated control group 3 (n = 2). After 5-day acclimatization, the pigs in groups 1 and 2 were challenged intranasally (1 ml per nostril) with a 2-ml dose of LOM vaccine or KNU-1905 virus at a 50% tissue culture infectious dose per milliliter ($TCID_{50}$/ml) of $10^{4.0}$, respectively. The pigs from group 3 were sham-inoculated with cell culture media as a placebo. Following inoculation, all animals were monitored daily for clinical signs throughout the experiments. Rectal temperature and body weight were recorded at 0, 5, 7, 14, 21, and 28 days post-inoculation (DPI). Blood was taken from pigs in all groups at 0, 5, 7, 10, 14, 21, and 28 DPI, and serum samples were centrifuged. Nasal and fecal samples from pigs in all groups were also collected with 16-inch cotton-tipped swabs at the same interval and were prepared as 10% (wt/vol) suspensions, as described previously (Jang et al., 2021; Lee et al., 2015). All pigs from the virus-infected and sham-infected groups were euthanized and necropsied at 28 DPI. Various organ specimens (submandibular lymph node, mesenteric lymph node, inguinal lymph node, tonsil, lung, liver, spleen, kidney, duodenum, jejunum, ileum, and colon) were collected at necropsy and prepared as 10% (wt/vol) homogenates, as described previously (Lee et al., 2015). The animal experiments described here were carried out in accordance with the guidelines established by the Institutional Animal Care and Use Committee.

3.3. Clinical examinations

A clinical significance score (CSS) was determined using the

previously established scoring system (Mittelholzer et al., 2000) with minor modifications to define the virulence of CSFV strains under identical experimental conditions. The determination of CSS values was based on seven CSF-relevant clinical manifestations, including lethargy (i.e., anorexia or depression), fever (pyrexia), skin hemorrhage (or cyanosis), conjunctivitis, respiratory symptoms (e.g., dyspnea, sneezing, and nasal discharge), diarrhea (or constipation), and neurological symptoms (e.g., tremors and ataxia). Each clinical presentation was judged as 1 point, and scores from each manifestation were added up, resulting in the total CSS of individual animals ranging from 0 to 7.

3.4. Quantitative real-time RT-PCR (qRT-PCR)

RNA isolation from serum, nasal, fecal, and tissue samples was performed automatically using an SLA-E13200 TANBead Nucleic Acid Extraction System (Taiwan Advanced Nanotech, Taoyuan, Taiwan) with a TANBead Nucleic Acid Extraction Kit (Taiwan Advanced Nanotech), following the manufacturer's recommendations. CSFV 5′-UTR-based qRT-PCR was performed using a VDx CSFV qRT-PCR kit (MEDIAN Diagnostic, Chuncheon, South Korea), in accordance with the manufacturer's instructions. The reaction was performed using a Thermal Cycler Dice Real-Time System (TaKaRa, Otsu, Japan) according to the manufacturer's protocols under the following conditions: 1 cycle of 50°C for 30 min, 1 cycle of 95°C for 15 min, and 42 cycles of 95°C for 10 sec and 60°C for 1 min. The results were analyzed using an automatic baseline, as described previously (Lee et al., 2017; Sagong and Lee, 2011). A CSFV strain (LOM vaccine) with a known infectivity titer was 10-fold serially diluted to

generate a standard curve for each PCR plate. The virus concentrations (genomic copies/ml) in the samples were calculated based on this standard curve. The mean cycle threshold (*Ct*) values were calculated based on PCR-positive samples, and the mean virus titers were calculated based on all pigs within the group.

3.5. CSFV serology

CSFV E2-specific antibodies in serum samples collected from pigs experimentally inoculated with each virus or shame-inoculated were detected by the commercially available CSFV antibody B-ELISA kit (BIONOTE, Hwaseong, South Korea) according to the manufacturer's instructions. The results were expressed as the percent inhibition and a percent inhibition value equal to or greater than 40 was considered positive for the presence of CSFV antibodies.

3.6. Statistical analysisy

All values are expressed as mean ± standard deviation of the mean difference (SDM). Statistical analyses were conducted using the GraphPad Prism 7 software package (GraphPad Software, San Diego, CA). *P*-values below 0.05 were considered to be statistically significant.

4. Results

4.1. Pathogenicity of the LOM vaccine and LOM-derived field isolate KNU-1905 strains in pigs

To assess the potential for reversion to or increase in virulence of the commercial LOM vaccine in target animals, the pathogenicity of the LOM and its field derivative KNU-1905 strains were characterized in pigs. Ten pigs, divided into three groups of four animals each, were challenged intranasally with KNU-1905 (group 1) or LOM (group 2), and the remaining two pigs in a control group were sham inoculated with cell culture media (group 3). Clinical signs were recorded daily before and after the challenge for the duration of the study. During acclimation, all animals were active and showed no clinical manifestations. Following the challenge, none of the control pigs in group 3 developed clinical abnormalities associated with CSFV infection. Although two or three of the pigs infected with the LOM strain showed mild respiratory signs, including nasal discharge or sneezing, at 5–7 DPI (mean CSS of 0.5–0.75), all the animals in group 2 maintained a good health condition without remarked clinical presentation, similar to the control group (Fig. 1A). By contrast, KNU-1905-challenged pigs (group 1) exhibited multiple clinical signs accompanied by lethargy, conjunctivitis, nasal discharge, and diarrhea at 5–21 DPI and had significant CSS values (mean CSS of 0.75–2.50) compared to the LOM-inoculated pigs (group 2). In addition, the virus-infected pigs in groups 1 and 2 underwent neither CSF-specific nervous symptoms nor death.

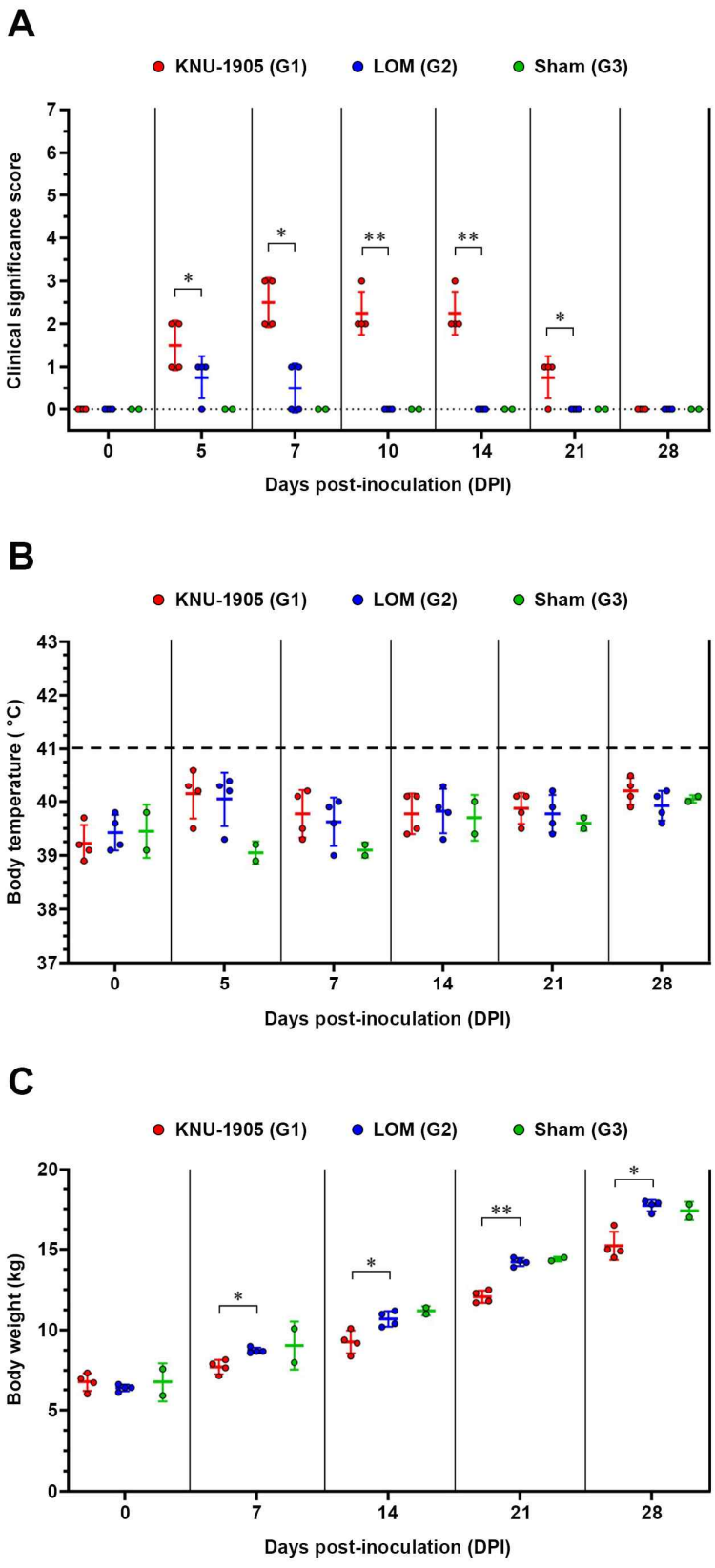

Figure 1. Clinical significance scores (A), rectal temperatures (B), and body weights (C) in pigs from three experimental groups: KNU-1905-inoculated group 1 (G1), LOM vaccine-inoculated group 2 (G2), and mock-inoculated control group 3 (G3). Clinical significance scores were measured as described in the Materials and Methods section. Rectal temperatures ≥41°C (indicated as the dashed line) were defined as a high fever. Error bars represent the SDM. *P*-values were calculated by comparing results from the KNU-1905-inoculated group 1 and LOM-inoculated group 2 using GraphPad Prism software. *, $P < 0.05$; **, $P < 0.001$.

None of the animals in all groups had a high fever (> 41°C) throughout the experiment. Overall, the virus-infected groups 1 and 2 had a febrile response similar to the sham-infected control group and within the normal range, despite slightly higher mean rectal temperatures in these groups than in group 3 at 5 and 7 DPI (Figure 1B). All pigs were weighed at a 7-day interval, and the average body weight in each group was plotted at the indicated time points (Figure 1C). There was no noteworthy difference between the average weight gains of the LOM-inoculated and sham-inoculated groups at the indicated period; the pigs in groups 2 and 3 gained average weights of 11.29 and 10.65 kg, respectively, during the observation period (28 days). By contrast, as shown in Figure 1C, pigs in group 1 infected with KNU-1905 gained significantly less body weight than those in groups 2 and 3, achieving an average weight of 8.47 kg throughout the experimental period.

The serum samples collected from the pigs were used for the detection of CSFV RNA (viremia) and seroconversion. CSFV RNA was not detected by qRT-PCR in any of the sham-infected pigs (group 3), and the pigs remained CSFV-negative throughout the study (Figure 2A). In group 2, viral RNA was detected from the sera of two animals (2/4) inoculated with the LOM vaccine strain (Table 1): one pig was intermittently viremic at 7 and 14 DPI with a viral RNA load of $10^{0.75}$ and $10^{2.22}$ copies/ml, respectively, and another pig was transiently viremic at 10 DPI with a viral RNA load of $10^{3.56}$ copies/ml (Figure 2A). However, CSFV RNA was detected in the KNU-1905 strain-infected pigs (4/4) from 5 DPI and continuously present in their sera until 21 DPI, except for one that was

viremic up to 14 DPI (Table 1). The mean viral RNA loads in these pigs ranged from $10^{3.60}$ to $10^{7.62}$ copies/ml (at 21 and 10 DPI, respectively), values greater than in the LOM-inoculated pigs (Figure 2A). Detection of the presence of CSFV E2-specific antibodies by ELISA showed that the placebo-infected control pigs remained seronegative to CSFV throughout the trial. Interestingly, only two pigs (2/4) in the group inoculated with the LOM vaccine strain seroconverted by 21 DPI, whereas the remaining animals were seronegative until the end of the experiment. Conversely, all pigs infected with KNU-1905 in group 1 seroconverted by 21 DPI (seroconversion occurred in two pigs by 14 DPI) (Figure 2B). The antibody titers presented as percent inhibition values for the KNU-1905-infected group continued to rise gradually and were higher than those for the LOM-infected group.

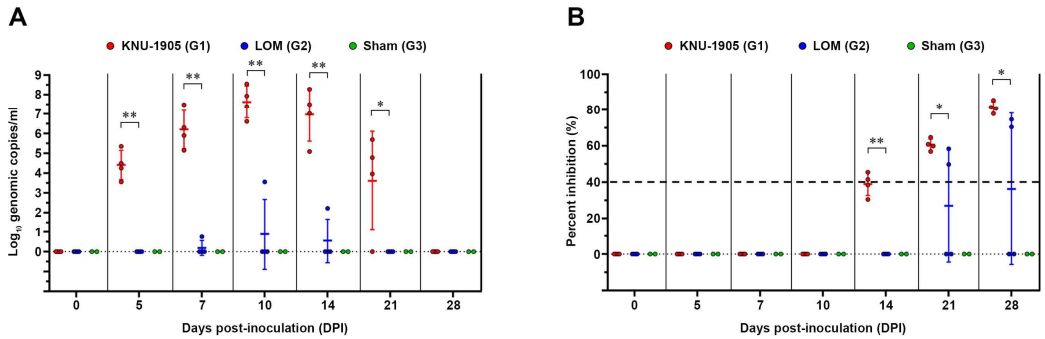

Figure 2. Viremia and seroconversion in pigs from three experimental groups: KNU-1905-inoculated group 1 (G1), LOM vaccine-inoculated group 2 (G2), and sham-inoculated control group 3 (G3). (A) CSFV antigen levels of pigs. Quantification of CSFV genomic RNA in serum samples at each time point was determined using real-time RT-PCR analysis. The virus titers were expressed as genomic copies/ml. (B) CSFV E2-specific antibody response of pigs as measured by ELISA. Samples are considered positive for antibodies to CSFV if the percent inhibition value is equal to or greater than 40 indicated as the dashed line. Error bars represent the SDM. *P*-values were calculated by comparing results from the KNU-1905-inoculated group 1 and LOM-inoculated group 2 using GraphPad Prism software. *, $P < 0.05$; **, $P < 0.001$.

Table 1 Detection of CSFV RNA in pigs inoculated with the LOM vaccine or LOM-derived Jeju isolate

	Inoculum strain	No. of pigs	Sample	No. of CSFV positive pigs/ No. of pigs tested						
				0[a]	5	7	10	14	21	28
Group 1	KNU -1905	4	Serum	0/4	4/4	4/4	4/4	4/4	3/4	0/4
			Nasal	0/4	3/4	4/4	4/4	4/4	1/4	0/4
			Fecal	0/4	2/4	3/4	4/4	3/4	0/4	0/4
Group 2	LOM	4	Serum	0/4	0/4	1/4	1/4	1/4	0/4	0/4
			Nasal	0/4	0/4	0/4	1/4	0/4	0/4	0/4
			Fecal	0/4	0/4	0/4	0/4	0/4	0/4	0/4
Group 3	Control	2	Serum	0/2	0/2	0/2	0/2	0/2	0/2	0/2
			Nasal	0/2	0/2	0/2	0/2	0/2	0/2	0/2
			Fecal	0/2	0/2	0/2	0/2	0/2	0/2	0/2

[a] Days post-inoculation

All animals were euthanized and necropsied at the end of the study for post-mortem assessments to evaluate the presence of pathological symptoms in different organs and tissues. No lesions were observed in the organs collected from the LOM-inoculated pigs in group 2, which were macroscopically comparable to the sham-infected control group (Figure 3, middle and bottom panels). However, from a macroscopic pathology perspective, all the pigs infected with the LOM-derived KNU-1905 strain (group 1) presented mild-to-moderate CSF-specific pathological changes, particularly in immune organs, including enlargement and hemorrhage of the lymph nodes and hemorrhagic infarction around the edge of the spleen (Figure 3, top panels).

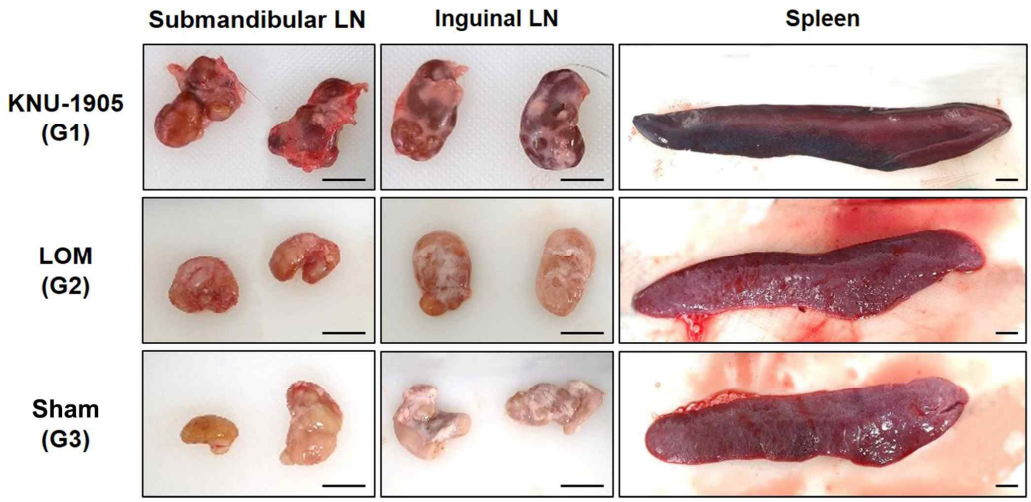

Figure 3. Representative macroscopic lesions in pigs from three experimental groups: KNU-1905-inoculated group 1 (G1), LOM vaccine-inoculated group 2 (G2), and sham-inoculated control group 3 (G3). All experimental pigs were euthanized at 28 DPI, and organs were collected and examined for gross lesions. Note that pigs infected with KNU-1905 (top panels) show enlargement and hemorrhage in the lymph nodes and spleen compared to LOM- or mock-infected pigs (middle and bottom panels). Scale bars = 10 mm.

4.2. CSFV shedding and tissue distribution in pigs infected with the LOM vaccine or KNU-1905 strain

Nasal and rectal swabs were collected at the indicated time points and subjected to qRT-PCR to quantify viral RNA for viral shedding analysis. CSFV RNA was not detected in the nasal fluid and feces of any negative-control pigs tested throughout the experimental period. Similarly, except for one pig (1/4) with viral shedding in the nasal fluid at 10 DPI, most animals inoculated with the LOM vaccine strain in group 2 had no detectable CSFV-shedding in the nasal fluid and feces during the study (Table 1). However, in the KNU-1905-infected group, CSFV RNA was identified in 3/4 nasal secretions at 5 DPI, 4/4 at 7–14 DPI, and 1/4 at 21 DPI (Table 1), with a mean viral shedding of $10^{0.91}$–$10^{5.06}$ genomic copies/ml during 5–21 DPI (Figure 4A). Viral RNA was also detected in 2/4 fecal samples at 5 DPI, 3/4 at 7 DPI, 4/4 at 10 DPI, and 3/4 at 14 DPI, with a mean viral shedding of $10^{0.89}$–$10^{4.71}$ genomic copies/ml during 5–14 DPI (Figure 4B).

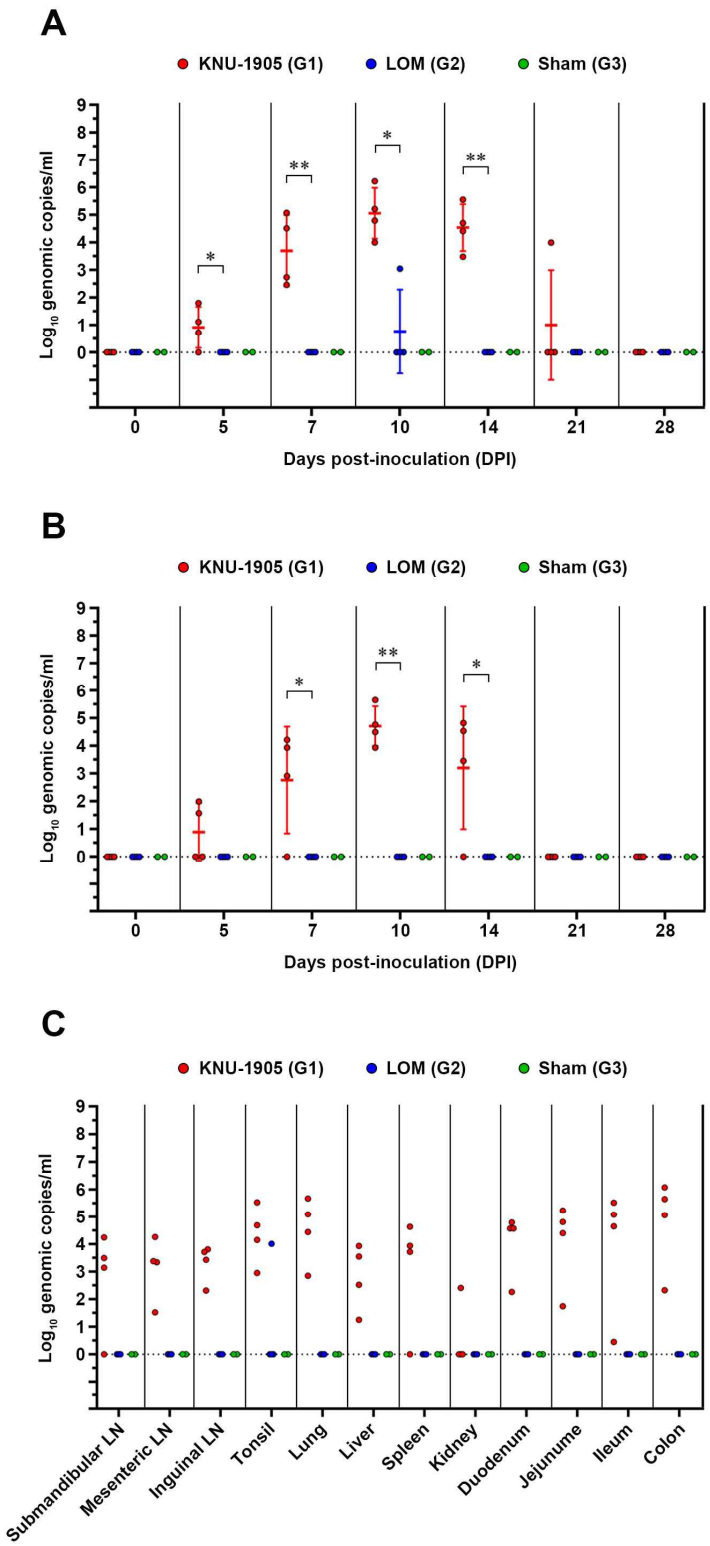

Figure 4. CSFV RNA detection in nasal (A), fecal (B), and tissue (C) samples of pigs from three experimental groups: KNU-1905-inoculated group 1 (G1), LOM vaccine-inoculated group 2 (G2), and mock-inoculated control group 3 (G3). Virus shedding in nasal and rectal swabs and viral loads in the indicated tissues were determined using real-time RT-PCR analysis. The virus titers were expressed as genomic copies/ml. Error bars represent the SDM. *P*-values were calculated by comparing results from the KNU-1905-inoculated group 1 and LOM-inoculated group 2 using GraphPad Prism software. *, $P < 0.05$; **, $P < 0.001$.

In addition, each tissue sample collected from the pigs euthanatized at 28 DPI was tested for the presence of CSFV RNA by qRT-PCR. As expected, there was no detectable CSFV RNA in organs from sham-infected pigs in group 3, whereas viral RNA was present only in tonsil collected from one pig infected with LOM in group 2 (Figure 4C). However, the KNU-1905-infected pigs had considerably high viral loads in all tissue samples tested, including lymph nodes, tonsil, lung, liver, spleen, kidney, and intestines. In particular, CSFV RNA was detectable in 100% (4/4) of lymph node (mesenteric and inguinal), tonsil, lung, liver, and intestinal (duodenum, jejunum, ileum, and colon) samples taken from all of the pigs in group 1. Individual viral loads in various tissues sampled after necropsy for pigs inoculated with the LOM or KNU-1905 strain are further summarized in Table 2.

Table 2 Tissue distribution of CSFV RNA in pigs inoculated with the LOM vaccine or LOM-derived Jeju isolate

	Inoculum strain	No. of pigs	Viral loads in organs[a] of pigs (Log_{10} genomic copies/ml)											
			SL	ML	IL	To	Lu	Li	Sp	Ki	Du	Je	Il	Co
Group 1	KNU -1905	1	4.2	1.5	3.4	5.5	5.7	3.9	4.6	-	4.6	4.4	5.5	6.1
		2	0	3.3	3.8	4.2	5.1	3.6	-	-	4.6	5.2	4.7	5.7
		3	3.5	4.3	3.7	4.7	2.9	1.3	3.9	-	4.8	4.8	5.1	5.1
		4	3.1	3.4	2.3	3.0	4.4	2.5	3.7	2.4	2.3	1.7	0.5	2.3
Group 2	LOM	1	-	-	-	4.0	-	-	-	-	-	-	-	-
		2	-	-	-	-	-	-	-	-	-	-	-	-
		3	-	-	-	-	-	-	-	-	-	-	-	-
		4	-	-	-	-	-	-	-	-	-	-	-	-
Group 3	Control	1	-	-	-	-	-	-	-	-	-	-	-	-
		2	-	-	-	-	-	-	-	-	-	-	-	-

[a] SL: submandibular lymph node, ML: mesenteric lymph node, IL: inguinal lymph node, To: tonsil, Sp: spleen, Lu: lung, Li: liver, Sp: spleen, Ki: kidney, Du: duodenum, Je: jejunum, Il: ileum, Co: colon

5. Discussion

Since its first reported outbreak in 1947, CSF has been regarded as one of the most devastating diseases affecting intensive pig production in South Korea. The MLV-LOM was introduced in 1974 and has since been used nationwide to combat CSF in South Korea. In 1996, the implementation of a national CSF eradication program, including compulsory vaccination, was launched by the South Korean government, leading to a gradual reduction in the number of CSF outbreaks. As a result, the government declared in December 2001 that the country was CSF-free and CSF vaccination was stopped (Kim et al., 2008; Wee et al., 2005). Shortly afterward, recurrences of CSF occurred, with 13 (regional-scale) and 72 (national-scale) outbreaks reported in 2002 and 2003, respectively (Kim et al., 2008). Subsequent government policy changes have mandated vaccination and quarantine across the country since 2009 (Kim et al., 2008). However, Jeju Province was exempt because the provincial authority declared a CSF-free status and discontinued CSF vaccination in 1998 (Song et al., 2013). Although this region is considered free of the disease without vaccination, it may remain under a constant treat of the reintroduction if CSF-vulnerable pigs are exposed to the virus or even to the vaccine. This risk has become a reality, leading to various degrees of CSF resurgence in unvaccinated swine herds on Jeju Island for the past two decades through accidental exposure (or vaccination) of naïve pigs to the LOM vaccine. In particular, the MLV-LOM causing the last reemergence in 2014 continues to ravage the provincial swine industry and has continued to undergo substantial genetic variations in the field, which

may contribute to reversion to an original low virulence phenotype of the commercial LOM vaccine strain (Jang et al., 2019, 2020). Therefore, I attempted to analyze the pathogenicity of the LOM-derived CSFV variant (KNU-1905) isolated in a CSF-recurrent pig farm on Jeju Island (Jang et al., 2020).

Adverse effects of the MLV-LOM have been reported in CSFV-naïve pregnant sows and immunosuppressed pigs co-infected with both PRRSV and PCV2 after LOM vaccination; the former case caused abortion (Choe et al., 2020; Lim et al., 2016a), while the latter was associated with a prolonged duration of viral shedding (Lim et al., 2016b). Nevertheless, a recent safety study revealed that despite the presence of CSFV RNA in some blood and organ specimens, specific pathogen-free (SPF) pigs inoculated with the LOM vaccine strain remained asymptomatic without CSFV RNA shedding in the stool or saliva during the experimental period (Choe et al., 2020). The present study also confirmed that LOM vaccination had no harmful influence on commercial pigs exhibiting the overall CSS, fever response, weight gain, and pathological lesions akin to unvaccinated control pigs; albeit some vaccinated pigs developed only mild and transient clinical signs. Although CSFV RNA was detected in serum and nasal samples from the same animal in group 2, the viral RNA detection outcomes in the blood, feces, or nasal fluid from the LOM-inoculated pigs were comparable to those previously reported (Choe et al., 2020). Unlike the previous study showing viral antigen detection in multiple organs, including tonsil, lymph nodes, spleen, and ileum, the present experiment detected CSFV RNA only in tonsil from one LOM-inoculated pig, whose blood and nasal secretion also contained viral

antigen. Choe et al. (2020) also reported that seroconversion occurred appreciably at 14 DPI in all animals following LOM inoculation; however, only 50% of the LOM-inoculated pigs seroconverted at 21 DPI under the current experimental condition. This dissimilarity may be explained by differences in the inoculation route of the LOM vaccine used in the experiments; the former study applied intramuscular (IM) inoculation that is identical to a common administration route of the MLV-LOM specified by the vaccine manufacturers, whereas the current study used intranasal (IN) inoculation, which is a natural infection and transmission route in the field. It is thus reasonable that the mucosal IN route might elicit a lower immune response in the host than the IM vaccination route.

The previous safety study also assessed the pathogenic characteristics of LOM-derived field (designated Jeju LOM) strains isolated in 2016. The consequences of experimental infection with the Jeju LOM strains were similar to those of the MLV-LOM infection, resulting in adverse effects on the fetuses of pregnant sows or no pathogenicity in SPF pigs (Choe et al., 2020). By contrast, a LOM-derived Jeju strain, KNU-1905, tested in the current study, caused clinical illness in infected young pigs, evident by notably higher CSS and less weight gain than LOM-vaccinated and control animals. Although previous work and the present findings commonly confirmed viremia in pigs during the course of infection, KNU-1905 induced a higher magnitude and longer duration viremia than the Jeju LOM viruses described by Choe et al. (2020). Similarly, the tissue distribution of CSFV RNA was comparable in each study, but the viral loads in immune organs, including lymph nodes and spleen, with macroscopic lesions determined in this study were greater than those in organs without

pathological changes in the previous study. These results further suggest that CSFV titer in individual organs would be positively associated with the severity of tissue injury. More interestingly, the viral-shedding pattern observed under the current experimental condition showed considerable durations and amounts of viral shedding in nasal secretions and feces of KNU-1905-infected pigs, in contrast to the previous data in which viral shedding in the saliva or feces of infected SPF pigs was absent (Choe et al., 2020). These clinically altered outcomes might be due to a discrepancy between the pig breeds (i.e., CSFV-naïve conventional versus SPF pigs) used in individual studies. Moreover, the virus strain used cannot be excluded because the LOM-derived field strain (KNU-1905) possesses unique genotypic features, including the INDEL and point mutations in the 3′-UTR or/and at other hotspots, which were absent in the Jeju LOM strains. In particular, genetic drift, including the INDEL and substitutions in the 3′-UTR, of the LOM field isolates might be associated with a reversion to the primary pathogenicity of LOM (Jang et al., 2020). Despite its unimportance for viral replication, the 3′-UTR in the CSFV plays an important role in viral pathogenesis (Li et al., 2014). Furthermore, the polyuridine insertion was reported in the 3′-UTR of several attenuated vaccine or low virulence strains of CSFV, indicating its function in virulence (Coronado et al., 2017; Fan et al., 2008; Wu et al., 2001; Wang et al., 2020). Thus, gain-of-function research using reverse genetics is needed to investigate whether clinical and pathogenic characteristics of KNU-1905 described in this study stem from its genetic evolution in hotspots, including the 3′-UTR, or are identical to those of the LOM itself.

CSFV infection results in a failure in the immune system, such as

lymphopenia, accompanied by a so-called cytokine storm due to aberrant upregulation of inflammatory cytokines and chemokines, which is incompetent to control disease progression (Ganges et al., 2020). Although I did not measure leukocyte and cytokine concentrations in this study, SPF pigs infected with the Jeju LOM strains have temporarily shown lymphopenia with elevated interleukin-10 levels related to general immunosuppression (Choe et al., 2020). This situation may be connected to secondary bacterial infection in the LOM-affected pig farms, which can aggravate damage in the field. Another concern could be a practical co-infection scenario of the LOM virus with immunosuppressive pathogens, such as PRRSV and PCV2, despite infection with multiple viruses (MLV-LOM, PRRSV, and PCV2) shown to be irrelevant to any adverse effects in pigs (Lim et al., 2016a). However, the MLV-LOM can remain in the immunosuppressed pigs for a relatively long time, amplifying the risk of virus shedding and transmission (Lim et al., 2016a). Furthermore, due to the continuing circulation of various PRRSV and PCV2 strains across the province, the viruses are endemic in Jeju swine populations (Jang et al., 2021). Considering these circumstances, the possibility that co-infection of LOM virus with PRRSV or PCV2 or both may occur can increase and, consequently, may lead to serious clinical outcomes in the field at any time. More importantly, the pigs infected with the LOM-derived KNU-1905 strain could shed large amounts of the virus in their nasal fluids and feces for more than a week after infection, which serve as a critical source for horizontal transmission. Indeed, most contemporary CSFV detection and isolation cases in Jeju herds have been identified on farms with recurrent LOM infections (Jang et al., 2020). In addition, I detected a high viral

load in the tonsils of all the pigs infected with KNU-1905 or even in that of the LOM-inoculated pig at 28 DPI. This result indicates that the tonsil is a persistent infection site for the LOM strain, which may be relevant to viral shedding in oral fluids. Given these conditions, animals that have been LOM-infected but survived can remain asymptomatic or mild-symptomatic and become PI pigs that can exude an infective quantity of the virus in their saliva, nasal secretions, and feces and act as dangerous virus reservoirs to spread the virus via pig-to-pig and farm-to-farm transmissions. Therefore, the diagnosis and subsequent pre-emptive stamping out of PI shedders from affected farms must be proactively practiced in parallel with the efforts to regain the former CSFV-free status on Jeju Island.

The MLV-LOM has remained the sole national vaccine used to immunize pigs in South Korea since 1974. Although its adverse effects are questionable owing to the main drawback of using the MLVs that can potentially revert to a virulent wild-type strain, the LOM vaccine policy has been sustained to eliminate CSF in mainland South Korea, excluding Jeju Island. However, the safety issue of the MLV-LOM was reignited because the LOM strain that was incidentally introduced onto CSF-free Jeju Island in 2014 has affected Jeju herds and undergone adaptive genetic evolution (Jang et al., 2019, 2020; Je et al., 2018). The present study reports the pathogenic characterization of a novel LOM variant with the 3′-UTR INDEL isolated from the pig farm with a recurrent LOM-associated CSF outbreak on Jeju Island. Our data provide evidence that the LOM-derived field virus is a pathogenic revertant of the MLV-LOM, likely resulting from phenotypic changes involved in viral fitness due to substantial genetic

changes acquired during field adaptation (since 2014). It is presumed that the LOM variants are capable of establishing persistent or chronical infection under the endemic disease circumstance, without including severe clinical manifestations that would allow easy detection and elimination in field farms. In conclusion, the LOM-derived CSFV appears to evolve genetically and clinically in Jeju herds, leading to its phenotype shift from the vaccine itself to the disease-causing virus. To this end, continuous monitoring and surveillance of the LOM virus are an indispensable practice to clarify the characteristics of the LOM variants in the field, and customized control strategies based on individual farm circumstances, including biosecurity practices, vaccination, PI pig removal, and sow replacement (if possible), should be established and implemented to control CSF on this LOM-contaminated island.

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Abstract in Korean

돼지열병 바이러스 LOM 제주 야외주의 유전학적 특성 및 병원성 연구

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돼지열병 비백신 청정지역인 제주도내 사육돼지에서 2014년도에 돼지열병 백신항원(LOM주)이 검출되고 검출모돈에서 유·사산이 나타났으며, 어린 자돈의 경우 결막염, 수양성 설사 그리고 심한 경우 폐사를 보였다. 역학조사 결과 백신제조회사의 과실로 돼지열병 항원이 오염된 돈단독백신이 농가에 공급되었음이 확인되었고, 이후 돈단독 백신을 접종하지 않은 농장에서도 백신항원이 검출되고 피해가 발생되어 백신항원에 대한 전파 가능성과 병원성 문제가 지속적으로 제기되었다.

본 연구에서는 2017~2018년에 제주 양돈농장의 임상시료에서 분리된 돼지열병 LOM주 유래 바이러스의 전체 유전체를 분석하였으며. 2019년도 제주 양돈농가에서 분리된 LOM주 유래 변이바이러스의 동물실험을 통하여 LOM주의 병원성 회복과 전파 가능성을 확인하였다.

분자학적 특성 분석 결과 LOM주와 LOM주 유래 야외주는 E2 유전자 기준 98.9%~99.7%, 전체 유전체 기준 98.7%~99.0%의 상동성을 보였으며, 뉴클레오타이드/아미노산 차이는 111/28에서 148/42까지 다양하게 나타났다. 이러한 변이는 유전체 전체에 널리 분포되어 있으며, 특히 비구조단백질에 축적되어 있는 것으로 확인하였다.

2019년 제주 양돈농가에서 3′-UTR에서 특이한 삽입/결손 돌연변이(INDEL)를 가진 돼지열병바이러스를 분리하여 전체 유전체 서열과 분자학적 특성을 조사하였다. 이 변이주들의 유전체 길이는 12,297~12,302 뉴클레오타이드로 LOM주와 비교 시 1개 뉴클레오티드 짧거나 1, 2, 또는 4개의 뉴클레오타이드가 길었다. 3′-UTR INDEL 변이주들는 LOM주와 다단백질과 전체 유전체에서 각각 98.8-99.3%, 98.3~98.6%의 상동성을 보였다. LOM주와 3′-UTR INDEL 변이주들의 유전자는 뉴클레오티드와 아미노산에서 각각 161-202개와 37-45개 차이를 나타내었다. 이러한 돌연변이는 전체 유전체에 광범위하게 분산되었고, 특히 NS2와 3′-UTR에 집중되었고, 이는 3′-UTR INDEL 변이주가 저병원성으로 복귀되어, 야외에서의 순환감염의 지속성을 유지하도록 진화된 것으로 생각된다.

3′-UTR INDEL 변이주 KNU-1905 동물실험 결과 시판되는 LOM주 백신을 접종한 돼지에서는 부작용이 나타나지 않았으나, KNU-1905 분리주를 접종한 돼지에서는 임상증상(무기력, 결막염, 콧물, 설사), 체중감소 등을 확인하였다.. LOM주를 접종한 돼지에서는 혈청, 코, 편도에서만 CSFV RNA가 검출된 것과 대조적으로 KNU-1905를 접종한 돼지에서는 바이러스혈증, 분변과 콧물에서 바이러스가 분비되고 다양한 조직에서 바이러스가 확인되었다. 결론적으로 LOM유래 분리주는 돼지에서 임상증상을 유발하며 상당한 양의 바이러스를 분비하였다.

본 연구를 통해 제주도에서 순환되고 있는 LOM유래 분리주의 유전적 변이를 확인하였으며, 이 변이가 LOM주의 원래 병원성을 회복하여 만성감염 또는 지속감염을 유발하고, 이러한 바이러스 배출은 돼지열병 비발생지역의 돼지들 간 또는 양돈농장 간 바이러스 전파의 위험성이 있을 것으로 사료된다.

주요어: 돼지열병바이러스, LOM주 유래 야외주, 변이, 병원성 회복, 제주