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Development of a marker vaccine against classical swine fever

（豚熱に対するマーカーワクチンの開発）

Huynh Tan Loc

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Abbreviations

AAALAC International	Association for Assessment and Accreditation of Laboratory Animal Care International
AG	adrenal grand
ANOVA	analysis of variance
ASFV	African swine fever virus
BDV	border disease virus
BES	N,N-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid
BSA	bovine serum albumin
BVDV	bovine viral diarrhea virus
cDNA	complementary deoxyribonucleic acid
cm	centimeter
CMI	cell-mediated immunity
CSF	classical swine fever
CSFV	classical swine fever virus
DIVA	differentiating infected from vaccinated animals
DNA	deoxyribonucleic acid
dpc	days post-challenge
dpi	days post-inoculation
dpv	days post-vaccination
EB	extraction buffer
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMEM	Eagle’s minimum essential medium
ER	endoplasmic reticulum

E^{rns}	envelope protein ribonuclease secreted
g	times gravity
h	hours
HR	high resolution
HS	horse serum
ICS	immunochromatographic test strip
ID	identification
IFN	interferon
IFN-α/β	interferon-alpha/beta
IFN-γ	interferon-gamma
IgG	immunoglobulin G
IPX	immunoperoxidase
kb	kilobase
kDa	kilodaltons
L	liter
LAV(s)	live attenuated vaccine(s)
LN	mesenteric lymph node
LOM	low virulence of Miyagi
M	protein molecular weight marker
mAb	monoclonal antibody
MES	2-(N-morpholino)ethane sulfonic acid
MDA	maternally derived antibody
mg	milligram
min	minutes
mL	milliliter
MLV(s)	modified live attenuated vaccine(s)

mm	millimeter
MOI	multiplicity of infection
NCBI	National Center for Biotechnology Information
nm	nanometer
NRPV	Norway rat pestivirus
NS	no sample
NTD	N-terminal domain
OD	optical density
ORF	open reading frame
P	passage
p	probability
PAPeV	pronghorn antelope pestivirus
PBMC(s)	peripheral blood mononuclear cell(s)
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PCV2	porcine circovirus type 2
PhoPeV	phocoena pestivirus
PNGase F	peptide N-glycosidase F
POC	point-of-care
PRRSV	porcine reproductive and respiratory syndrome virus
RNA(s)	ribonucleic acid(s)
RNase	ribonuclease
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
SD(s)	standard deviation(s)
SDS-PAGE	sodium dodecyl-sulfate polyacrylamide gel electrophoresis

SK-6 Mx/Luc	swine kidney-6 carrying a Mx/Luc reporter gene
SK-L	swine kidney line-L
SNT	serum neutralization test
SPF	specific pathogen-free
$TCID_{50}$	50% tissue culture infectious dose
TPB	tryptone phosphate broth
UTR(s)	untranslated region(s)
UV	ultraviolet
v/v	volume/volume
w/v	weight/volume
WOAH	World Organisation for Animal Health
YEP	yeast peptone extract
µF	microfarad
µg	microgram
µL	microliter
µM	micromolar

Notes

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Preface

Classical swine fever (CSF) is a contagious viral disease severely affecting domestic pigs and wild boars with high mortality. It poses a significant threat to pig populations worldwide, leading to severe economic losses, trade restrictions, and animal welfare concerns [1,2]. This disease is caused by the CSF virus (CSFV), a member of the *Pestivirus* genus within the *Flaviviridae* family. The phylogeny of CSFV is closely related to bovine viral diarrhea virus (BVDV) and border disease virus (BDV) as well as several additional species that are found as atypical pestiviruses [3,4]. The CSFV genome is a single-stranded positive-sense RNA of approximately 12.3 kb in length with an open reading frame (ORF) flanked by two untranslated regions (UTRs), 5′-UTR and 3′-UTR. The ORF encodes a polyprotein of approximately 4,000 amino acids, which is cleaved by cellular and viral proteases cotranslationally and post-translationally into twelve proteins, including four structural proteins (C, E^{rns}, E1, and E2) and eight non-structural proteins (N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [5,6]. Notably, envelope glycoprotein E2 is a prominent and protective antigen that can elicit neutralizing antibodies and is the primary target antigen of molecular and serological diagnostic assays used to assess vaccination effectiveness against CSFV [7,8]. Furthermore, E^{rns} can also induce the neutralizing antibody and the antibody response to E^{rns} can be used as a marker to diagnose CSFV infection in pigs [9–11]. In particular, the glycoprotein E^{rns} is unique to pestiviruses, lacking homologs even among closely related hepaciviruses or pegiviruses. This protein exhibits distinctive characteristics, including an unusual membrane anchor, secretion as a protein, and ribonuclease (RNase) activity [6].

Efforts spanning several decades have not sufficed to eradicate CSF from endemic and re-emerging regions [12–14]. Vaccination remains the primary strategy in CSF-endemic areas, exemplified by the usage of the GPE^- live attenuated vaccine (LAV) in Japan, resulting in CSF-free status by 2007 according to the World Organisation for Animal Health (WOAH). However, the 2018 CSF outbreak in Japan underscored the ongoing challenges despite decades of successful eradication programs [15,16]. LAVs lack a serological marker for differentiating infected from vaccinated animals (DIVA), complicating disease eradication verification and international trade. Modified live attenuated vaccines (MLVs) based on chimeric pestivirus concepts show promise by combining vaccine efficacy with serological markers. Recent advancements include the

development of marker vaccines against CSF, which were licensed in Europe and Korea [17,18]; however, the phylogenetic closeness between CSFV and BVDV can lead to cross-reactivity of the CSFV E^{rns} antibody, resulting in false positives and impeding DIVA recognition. To address this, chimeric viruses incorporating E^{rns} substitutions from genetically distant pestiviruses represent a promising approach to achieve the DIVA concept and enhance CSF eradication efforts [19,20].

In CSF-endemic countries, traditional control strategies using LAVs and subunit vaccines face challenges like immunosuppression from persistent CSFV infections and interference from maternally derived antibodies (MDA), reducing vaccine efficacy and causing sporadic outbreaks [21,22]. Effective serological antibody assessment through extensive screening is crucial for enhanced CSFV control. Current antibody evaluation methods, such as serum neutralization tests (SNT) and enzyme-linked immunosorbent assay (ELISA) [23,24], though sensitive and accurate, are time-consuming, costly, and requiring laboratory settings. Immunochromatographic strip test (ICS) offers a rapid, reliable, and cost-effective alternative for point-of-care (POC) diagnostic tool [25]. Thus, the development of an ICS with the DIVA represents a significant advancement in practical diagnostic tools for detecting CSFV antibodies. This innovation will strongly support efforts aimed at controlling and eradicating CSF in the field.

As mentioned above, vaccination using LAVs is crucial for controlling CSF outbreaks due to their effectiveness. However, they carry risks such as reversion to virulence [13,14,26,27]. Notably, the glycoprotein E^{rns}, a key component of pestiviruses, plays vital roles in viral replication and immune evasion through its RNase activities and is also a pivotal antigen in vaccine development. Chimeric pestiviruses incorporating E^{rns} from genetically related, non-CSF pestiviruses have been developed to enhance CSF marker vaccines with DIVA capabilities, yet comprehensive safety assessments, particularly concerning virulence reversion in pigs, remain inadequately addressed [18,19,28]. Therefore, a thorough investigation into the safety profiles of chimeric marker vaccine candidates is essential to evaluate their safety and suitability for CSF control and eradication strategies.

Thus, this study developed novel marker vaccine candidates for CSF and thoroughly evaluated their biological profiles and applicability to DIVA strategies. As

outlined in Chapter I, two chimeric viruses derived from the GPE$^-$ vaccine strain were generated and assessed for their efficacy in providing early protection to pigs against the CSFV challenge. Building on this development, Chapter II described the development of a dual ICS designed to detect antibodies against CSFV E2 and E^{rns}. This ICS ensured the DIVA properties of the abovementioned two chimeric vaccine candidates and their applicability in the field. In Chapter III, the safety profiles of the two chimeric marker vaccines were confirmed, particularly addressing concerns about reversion to virulence. By highlighting the importance of controlling and eradicating transboundary diseases like CSF, the outcomes may significantly contribute to the development of marker vaccines, thereby enhancing efforts to control and eradicate CSF.

Chapter I

Generation and efficacy of two chimeric viruses derived from GPE$^-$ vaccine strain as classical swine fever vaccine candidates

Introduction

Extensive vaccination using LAVs has been implemented and widely applied in several countries as a mandatory CSF control program, along with other biosafety approaches [24]. Although the advantage of the above vaccines is to provide complete protection against CSF, a drawback of vaccines is the lack of an immunological marker for DIVA strategies, making proof of CSF eradication difficult and inhibiting international pig trade [29,30]. In this scenario, the ideal vaccine can be engineered by combining LAVs with marker properties [29,31].

Several strategies for developing CSF marker vaccines have been broadly investigated, including MLV, viral vector vaccine, and subunit vaccine [20]. In this regard, the MLV based on the chimeric pestivirus concept was considered a promising genetic construction to enable the DIVA system by combining the efficacy of the LAV with a serological marker [29]. Several CSF marker vaccine candidates have recently been generated based on the phylogeny of closely related pestiviruses to CSFV [17,18,32,33]. On this subject, the chimeric vaccine harboring the E2 sequence of the CSFV Alfort/187 strain based on the backbone of the BVDV CP7 strain, namely CP_E2alf [17], and the mutant chimeric virus based on the backbone of the CSFV LAV LOM strain with the substitution of the complete BVDV E^{rns} sequence, namely Flc-LOM-BE^{rns} [18], have been licensed and approved as live marker vaccines in Europe and Korea, respectively [18,34]. Nevertheless, the cross-reactivity due to the genetically close relationship among pestiviruses could not satisfy the DIVA strategy [35,36]. Therefore, improvements in the chimeric virus's construction should be considered to overcome this drawback.

Considering the developments of CSF marker vaccine candidates and addressing the issue of chimeric pestiviruses closely related to CSFV, a scheme of chimera construction based on substituting viral glycoprotein E^{rns} has been employed to design chimeric pestiviruses that are genetically and antigenically distant from CSFV [19]. Consequently, three chimeric viruses, namely "Ra," "Pro," and "RaPro," have been generated by substituting the E^{rns} sequence of the CSFV Alfort-Tübingen strain with those of Norway rat pestivirus (NRPV) and pronghorn antelope pestivirus (PAPeV) or a combination of both, respectively [19]. However, several concerns about these chimeric viruses could arise with the use of the virulent CSFV strain as a backbone. As a result, the

viral genome was still detected in the organ tissues of several vaccinated pigs, indicating that additional attenuation is required to obtain a safe vaccine [19]. In this case, a combination of LAV strains as the backbone with a gene marker genetically and antigenically distant from CSFV would be novel for chimeric virus construction.

Previous studies have established the reverse genetic system for the GPE$^-$ vaccine strain, resulting in the generation of the vGPE$^-$ strain consisting of 10 amino acid substitutions compared to the GPE$^-$ vaccine seed [37–39]. In addition, an advanced study was conducted to investigate the effects of these 10 amino acid substitutions, indicating that the vGPE$^-$ primarily preserves properties comparable to the GPE$^-$ vaccine strain and is potentially valuable for developing a CSF marker vaccine [40]. In this regard, constructing chimeric viruses with marker properties based on the vGPE$^-$ strain backbone would aid in the development of novel CSF vaccine candidates with potential DIVA properties as well as advance the ongoing CSF eradication campaign in Japan.

This study was part of a series of research projects aimed at engineering the GPE$^-$ vaccine strain into a chimeric virus vaccine candidate possessing a potential immunological marker that may be used to develop a novel CSF marker vaccine in the future. By replacing the complete viral glycoprotein E^{rns} of the vGPE$^-$ strain with those of non-CSFV pestiviruses, either PAPeV or phocoena pestivirus (PhoPeV), two chimeric viruses, namely vGPE$^-$/PAPeV E^{rns} and vGPE$^-$/PhoPeV E^{rns}, were generated. The viral growth and genetic stability of the two chimeric viruses were evaluated *in vitro*. In addition, the optimal dose of the chimeric viruses to induce protection was also determined in pig experiments. The early-onset protection of these chimeric viruses was further evaluated in challenge studies with a moderately virulent CSFV strain 7 days after of vaccination.

Materials and Methods

Cells and viruses

The swine kidney line-L (SK-L) cell [41] was cultured in Eagle's minimum essential medium (EMEM; Nissui Pharmaceutical, Tokyo, Japan) supplemented with 0.295% tryptose phosphate broth (TPB; Becton Dickinson, Franklin Lakes, NJ, United States), 10 mM N,N-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES; Sigma-Aldrich, St. Louis, MO, United States), sodium bicarbonate (Nacalai Tesque, Kyoto, Japan), and 10% horse serum (HS; Thermo Fisher Scientific, Waltham, MA, United States). SK-L cells were used for viral production, titration, and serological tests. The cell line was incubated at 37 °C in 5% CO_2.

A recombinant clone of CSFV LAV, vGPE$^-$, was derived from pGPE$^-$ [37], and a plasmid containing its full-length cDNA was used. A CSFV vALD-A76 was also derived from the full-length cDNA clone of a virulent CSFV ALD-A76 strain, which was developed in a previous study [42].

Construction of chimeric pestiviruses

The chimeric viruses were constructed by substituting the complete E^{rns} sequence of vGPE$^-$ with that of non-CSFV pestiviruses, either PAPeV (GeneBank accession number NC02418.2) or PhoPeV isolate NS170386 (GeneBank accession number MK910229.1), which are genetically distant from CSFV. These two chimeric viruses were constructed based on the backbone of an infectious cDNA clone pGPE$^-$ [37] of a CSFV LAV GPE$^-$ using in-fusion cloning. PAPeV E^{rns} or PhoPeV E^{rns} insert fragments (each cDNA gene was synthesized by Fasmac Co., Ltd., Kanagawa, Japan) and pGPE$^-$ vector fragment were amplified by specific in-fusion polymerase chain reaction (PCR) primers using the KOD FX Neo (TOYOBO, Osaka, Japan) and the In-Fusion HD Cloning Kit (TaKaRa Bio, Shiga, Japan).

As the insertion of the PhoPeV E^{rns} sequence is toxic to bacteria, the plasmid of pGPE$^-$/PhoPeV E^{rns} could not be stably replicated in *Escherichia coli* (*E. coli*). Therefore, the full length of the vGPE$^-$/PhoPeV E^{rns} genome was constructed using the OriCiro® Cell-Free Cloning System [43] (Oriciro Genomics, Inc., Tokyo, Japan). The circular DNA of GPE$^-$/PhoPeV E^{rns} was constructed as described in the OriCiro® Cell-Free Cloning

System Manual version 4.1, released in January 2021. Briefly, the circular DNA of GPE^{-}/PhoPeV E^{rns} was constructed by applying the system consisting of two kits. In the first step, the OriCiro Assembly Kit allows the seamless assembly of six overlapping DNA fragments (forty nucleotides overlap), of which six fragments were generated by PCR using the KOD FX Neo. In the next step, the assembly product was directly added to the OriCiro Amp Kit for selective GPE^{-}/PhoPeV E^{rns} circular DNA amplification. The template was amplified by PCR (AccuPrime™ Taq DNA Polymerase System, Thermo Fisher Scientific) using a primer containing the T7 promoter sequence (TAATACGACTCACTATAG). The recovered cDNA with the target sequence was isolated using electrophoresis, followed by gel cutting and recovery. The full-length linear cDNA was confirmed by Sanger sequencing and used as a transcriptional template for virus rescue.

Virus rescue

The cDNA-derived chimeric viruses were rescued as described previously [22,29]. Briefly, the plasmid pGPE^{-}/PAPeV E^{rns} was linearized at the *Srf*I (a restriction endonuclease recognized the 8-base pair palindromic sequence correlating with 5'-GCCCGGGC-3' and cleaved double-stranded DNA following the third C in the sequence) site at the 3′-end of the viral genomic cDNA sequence and purified. The linearized product of pGPE^{-}/PAPeV E^{rns} and the full-length chimeric cDNA template of pGPE^{-}/PhoPeV E^{rns} were used for run-off transcription with the MEGAscript T7 kit (Thermo Fisher Scientific). After digestion with deoxyribonuclease I and purification on MicroSpin S-400 HR columns (Cytiva, Global Life Sciences Solutions Operations UK Ltd., Buckinghamshire, United Kingdom), RNA was quantified using NanoDrop One (Thermo Fisher Scientific) and transfected into SK-L cells by electroporation using a Gene Pulser Xcell Electroporation System (Bio-Rad, Hercules, CA, United States), set at 200 V and 500 μF, followed by incubation at 37 °C for 72 h. Immunoperoxidase (IPX) staining was performed to confirm virus recovery using an anti-NS3 monoclonal antibody (mAb) 46/1, as described previously [45].

Genetic stability assessment

The genetic stability of each virus was evaluated during blind passages [46]. The parental and chimeric viruses were passaged on SK-L cells for five rounds, and 100 μL

of the culture supernatants was used to infect naïve cells. At 72 h after inoculation, the culture supernatants were collected for viral titration and subjected to the next passage. The virus titers were determined in triplicate using SK-L cells. The cell culture supernatant of each passage was utilized for sequencing to confirm the genetic stability.

Sequencing

As described previously [37], the full-length cDNA clones of each chimeric virus and the entire genomes of rescued viruses were verified. Briefly, nucleotide sequencing of cDNA clones and PCR fragments from viral RNA was performed using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, United States) and an ABI 3500 Genetic Analyzer (Thermo Fisher Scientific). Sequencing data were then analyzed using the GENETYX® Network Edition version 15.0.1 software (GENETYX, Tokyo, Japan).

Virus titration

For virus titration, a serial 10-fold diluted viral stock was added to SK-L cells in 96-well plates. Cells were air-dried and heat-fixed at 80 °C after 96 h of incubation at 37 °C in 5% CO_2, followed by staining with anti-NS3 mAb 46/1 as the primary antibody for IPX staining [45]. The viral titers were calculated using the method of Reed and Muench and expressed as 50% tissue culture infective dose ($TCID_{50}$) per milliliter [47].

Animal use

Prophylactic vaccination in domestic pigs in Japan has been carried out since October 2019. The vaccination area has been expanded to most regions, excluding the island of Hokkaido. In this study, pigs were purchased from a CSFV-free farm in Hokkaido (Yamanaka Chikusan, Hokkaido, Japan) that had been proven to be free of antibodies against CSFV.

Animal experiments

To assess the pathogenicity of vGPE^{-}/PAPeV E^{rns} and vGPE^{-}/PhoPeV E^{rns}, five 2-week-old crossbred landrace × Duroc × Yorkshire specific pathogen-free (SPF) pigs (Yamanaka Chikusan) per independent experiment were inoculated intramuscularly with $10^{7.0}$ $TCID_{50}$ of each virus. All pigs were monitored daily for clinical scores according to

a scoring system for 14 days [48]. Blood and serum samples were collected in tubes containing ethylenediaminetetraacetic acid (EDTA) (Venoject II VP-NA050K; Terumo, Tokyo, Japan) and blood coagulation factor (Venoject II VP-P075K; Terumo), respectively, at 0, 3, 5, 7, 9, 11, and 14 days post-inoculation (dpi). The total numbers of leukocytes and thrombocytes were counted using a pocH-100iV Diff apparatus (Sysmex, Hyogo, Japan). All surviving pigs were euthanized at 14 dpi. The levels of CSFV-specific neutralizing antibodies in pigs at 0 and 14 dpi were evaluated via a serological test. Organ samples were collected aseptically, including tonsils, brains, spleens, adrenal glands, kidneys, mesenteric lymph nodes, and colons. For virus titration, 10% organ homogenates were prepared in 10% HS in EMEM and centrifuged at 3,000 rpm for 5 min. The virus titers were then measured and displayed as $TCID_{50}$/mL (blood) or $TCID_{50}$/g (tissue).

To evaluate the optimal infectious dose and immune responses of vGPE^{-}, vGPE^{-}/PAPeV E^{rns}, and vGPE^{-}/PhoPeV E^{rns}, nine 2-week-old crossbred landrace × Duroc × Yorkshire SPF pigs per independent experiment were inoculated intramuscularly with different doses of $TCID_{50}$. Three subgroups of three pigs were inoculated with $10^{3.0}$, $10^{4.0}$, and $10^{5.0}$ $TCID_{50}$ of the virus, respectively. All pigs were monitored daily for clinical scores according to a scoring system for 21 days [48]. Blood and serum samples were collected in tubes containing EDTA (Venoject II VP-NA050K; Terumo) and blood coagulation factor (Venoject II VP-P075K; Terumo), respectively, at 0, 7, 14, and 21 dpi. The total numbers of leukocytes and thrombocytes were counted by a pocH-100iV Diff apparatus (Sysmex, Hyogo, Japan). All pigs were euthanized at 21 dpi. The levels of CSFV-specific neutralizing antibodies of pigs at 0, 7, 14, and 21 dpi were evaluated by SNT. Virus yield was titrated and expressed as $TCID_{50}$/mL for blood samples.

The vaccine efficacy of each chimeric virus was evaluated. Due to the limitations of the present experimental conditions, vaccine efficacy studies were undertaken independently via two animal trials. In the first trial, nine 2-week-old crossbred landrace × Duroc × Yorkshire SPF pigs were randomly divided into three groups: the vGPE^{-} vaccination group ($n = 3$), vGPE^{-}/PAPeV E^{rns} vaccination group ($n = 3$), and control group ($n = 3$). In the second trial, six SPF pigs were divided into the vGPE^{-}/PhoPeV E^{rns} vaccination group ($n = 3$) and the control group ($n = 3$). These two trials were conducted under the same conditions: pigs in each vaccination group were intramuscularly injected with $10^{4.0}$ $TCID_{50}$ of vGPE^{-} or each chimeric virus on day 0, and pigs in each control

group were intramuscularly injected with 1 × phosphate-buffered saline (PBS; pH 7.4). The serum samples were collected at 0 and 7 days post-vaccination (dpv) to detect CSFV-specific neutralizing antibodies. At 7 dpv, pigs were intranasally inoculated with $10^{6.0}$ $TCID_{50}$ of the moderately virulent CSFV vALD-A76 strain and monitored daily for body temperature and clinical scores [48]. All surviving pigs were euthanized at 14 days post-challenge (dpc). Organ samples were collected aseptically, including tonsils, brains, spleens, adrenal glands (AG), kidneys, mesenteric lymph nodes (LN), and colons. For virus titration, the 10% organ homogenates were prepared in 10% HS in EMEM and centrifuged at 3,000 rpm for 5 min. The virus titers were then measured and displayed as $TCID_{50}$/mL (blood) or $TCID_{50}$/g (tissue).

Serum neutralization test

The SNT was conducted using a luciferase-based assay, as previously described [49]. In brief, equal volumes of serum and 100 $TCID_{50}$ of CSFV vGPE^{-}/HiBiT [46] were mixed well and incubated at 37 °C for 1 h. The mixture and SK-L cell suspension were then incubated in 96-well plates at 37 °C and 5% CO_2 for 96 h. Neutralizing antibody titers were then determined by the luciferase assay using the Nano-Glo HiBiT Lytic Detection System (Promega, Madison, WI, United States) and POWERSCAN®4 (Agilent Technologies International Japan Ltd., Tokyo, Japan).

Isolation of porcine peripheral blood mononuclear cells (PBMCs)

Whole blood was collected in Venoject II VP-CA050K70 (Terumo) from all pigs at 7 dpv. PBMCs were prepared from whole blood by density gradient centrifugation using Ficoll-Paque PLUS (Cytiva, Marlborough, MA, United States). Cells were finally resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum (Japan Bio Serum Co., Ltd., Hiroshima, Japan) and antibiotics (Meiji Seika Pharma Co., Ltd., Tokyo, Japan).

***In vitro* stimulation assay of PBMCs for the detection of CSFV-specific IFN-γ-secreting cells by enzyme-linked immunosorbent assay (ELISA)**

The *in vitro* stimulation assay of PBMCs was conducted following a previously described method [50]. Briefly, PBMC densities were determined and adjusted to 5×10^6 cells/mL, and 200 μL of PBMCs was transferred to wells of a 96-well V-bottom plate and

stimulated by 50 μL of medium containing the vGPE$^-$ strain at a multiplicity of infection (MOI) of 1. A mock inoculum prepared from an uninfected SK-L cell lysate was added in an equivalent volume to the negative control samples. Cells were incubated for 72 h at 37 °C in a humidified 5% CO_2 atmosphere, resuspended by pipetting, and centrifuged at 400 × *g* for 5 min. Cell-free supernatants were removed and immediately stored at −80 °C until analysis. IFN-γ was measured in the culture supernatants using an ELISA Flex: Porcine IFN-γ (HRP) Kit according to the manufacturer's instructions (Mabtech AB, Nacka Strand, Sweden). Absorbance at 450 nm was read using a BioTek Synergy H1 Multimode Microplate Reader (Agilent Technologies International Japan Ltd., Tokyo, Japan).

Statistical analysis

Statistical analyses of the data were performed using Student's *t*-test or one-way analysis of variance (ANOVA), followed by a post-test (Tukey's multiple comparisons), using GraphPad Prism version 9.5.1 (San Diego, CA, United States).

Ethics statement

Animal experiments were authorized by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine, Hokkaido University (approval number 18-0038, approved on 26 March 2018, and approval number 23-0029, approved on 23 March 2023) and performed according to the guidelines of this committee. Animals reaching the humane endpoint were euthanized by an intracardial injection of thiopental sodium (Ravonal®; Nipro ES Pharma Co., Ltd., Osaka, Japan) after deep sedation with isoflurane (Fujifilm Wako Pure Chemical Co., Ltd., Osaka, Japan). The experiment was conducted in animal facilities certified by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International).

Results

Rescue of chimeric viruses, *in vitro* characterization, and pathogenicity assessment in pigs

The full-length chimeric virus genomes were successfully constructed by substituting the E^{rns} sequence of the cDNA clone of CSFV vGPE$^-$ with that of PAPeV or PhoPeV (Figure 1a,b). The PAPeV E^{rns} was introduced into the vGPE$^-$ backbone to generate the infectious chimeric cDNA clone and the full-length linear cDNA of GPE$^-$/PhoPeV E^{rns} was constructed. Subsequently, the full-length chimeric viral RNA of vGPE$^-$/PAPeV E^{rns} or vGPE$^-$/PhoPeV E^{rns} was transfected into SK-L cells through electroporation resulting in the expression of a viral NS3 protein as shown by IPX staining. IPX staining confirmed that these two chimeric viruses effectively infected the monolayer SK-L cell culture with vGPE$^-$ used as the positive control and mock-transfected cells as the negative control (Figure 1c). Further investigations were performed *in vitro* to evaluate the viral growth and genetic stability of vGPE$^-$/PAPeV E^{rns} and vGPE$^-$/PhoPeV E^{rns} compared to the vGPE$^-$ strain by conducting blind passages for five rounds in the SK-L cells. Viral growth analysis demonstrated that vGPE$^-$/PAPeV E^{rns} and vGPE$^-$/PhoPeV E^{rns} exhibited high and stable replication efficiency ($>10^{7.0}$ $TCID_{50}$) in SK-L cells to a similar extent as vGPE$^-$ from the second passage (Figure 1d). In the next step, the vGPE$^-$/PAPeV E^{rns} and vGPE$^-$/PhoPeV E^{rns} genomes were sequenced to evaluate the genetic stability after serial passages. Both chimeric viruses displayed unchanged nucleotide sequences in the genome after five passages, indicating that vGPE$^-$/PAPeV E^{rns} and vGPE$^-$/PhoPeV E^{rns} maintained their efficient growth and genetic stability *in vitro*. In the next step, the chimeric virus vGPE$^-$/PAPeV E^{rns} or vGPE$^-$/PhoPeV E^{rns} was inoculated into pigs to assess the pathogenicity. As a result, no fever or clinical signs were found in all inoculated pigs (Figure 2a). Likewise, neither typical leukopenia nor thrombocytopenia was found in pigs inoculated with vGPE$^-$/PAPeV E^{rns} or vGPE$^-$/PhoPeV E^{rns} during the experiment (Figure 2b). In addition, no virus recovery was detected in blood or organ samples from all inoculated pigs (Tables 1 and 2). Notably, neutralizing antibodies were detected in almost all pigs at the end of the experiment, except in one vGPE$^-$/PhoPeV E^{rns}-inoculated pig with a neutralizing antibody of less than 1 (Table 1).

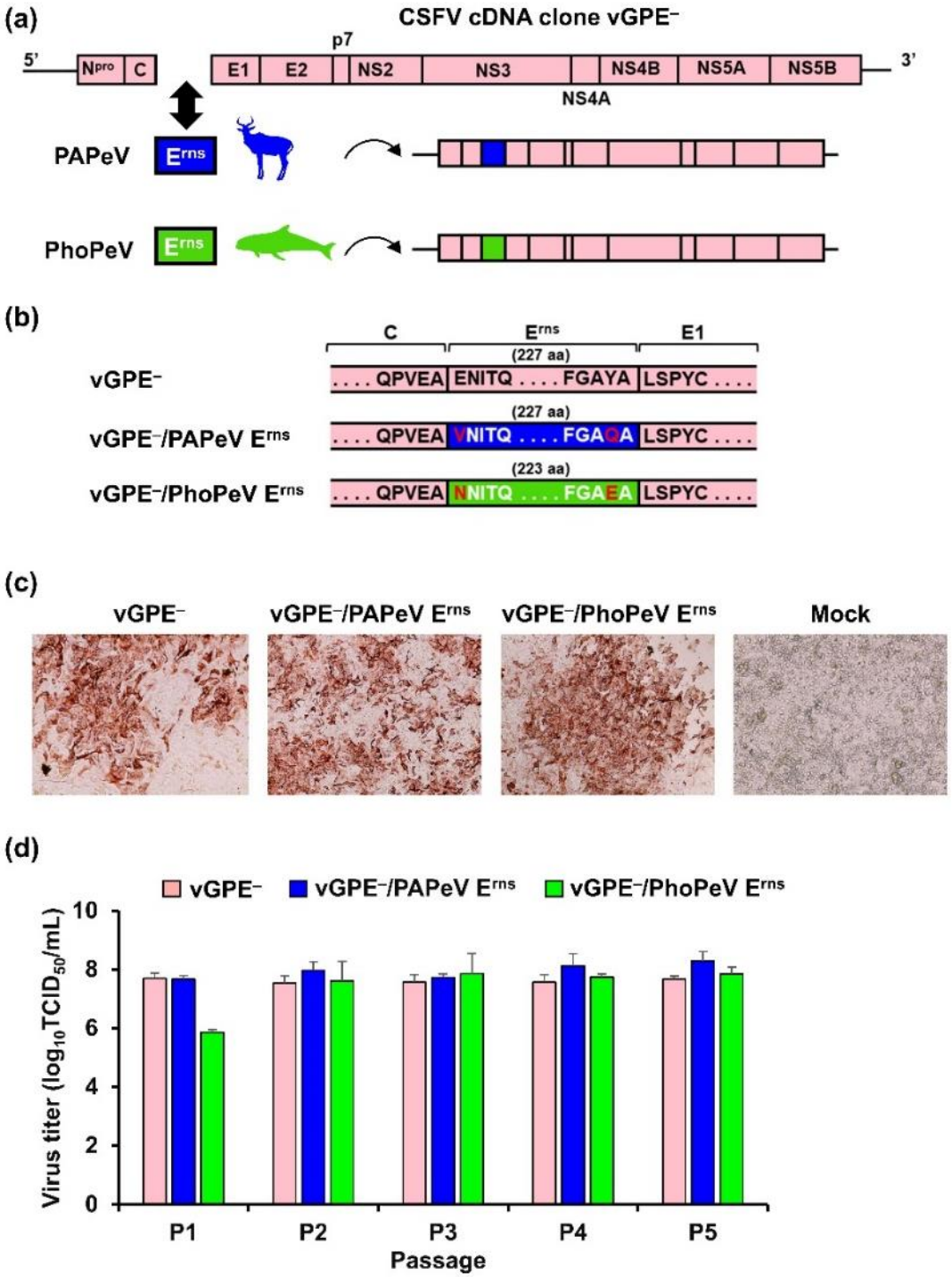


Figure 1. Construction and characterization of chimeric viruses *in vitro*. (**a**) The genomic structure of vGPE$^-$ and the design of chimeric viruses, vGPE$^-$/PAPeV E^{rns} and vGPE$^-$/PhoPeV E^{rns} contain the E^{rns} coding sequences of PAPeV and PhoPeV, respectively. (**b**) Partial amino acid sequence alignment of the E^{rns} sequence in the viral genome of vGPE$^-$/PAPeV E^{rns} or vGPE$^-$/PhoPeV E^{rns} compared to the parental vGPE$^-$.

The different amino acids are shown in red characters at the N-terminus and C-terminus of the PAPeV E^{rns} or PhoPeV E^{rns} protein compared to those of vGPE^{-}. (**c**) *In vitro*-transcribed RNAs derived from the viral cDNAs above were electroporated into SK-L cells. After 72 h, cells were heat-fixed and immunostained using anti-NS3 mAb 46/1. (**d**) Recombinant vGPE^{-}, vGPE^{-}/PAPeV E^{rns}, and vGPE^{-}/PhoPeV E^{rns} were passaged on SK-L cells for five rounds from the electroporated cells. After 72 h, the culture supernatants were collected and the virus titers were determined before inoculating them onto the next passage (P). The mean viral titers of the recombinant viruses in each passage are shown, with error bars representing standard deviations (SDs; $n = 3$).

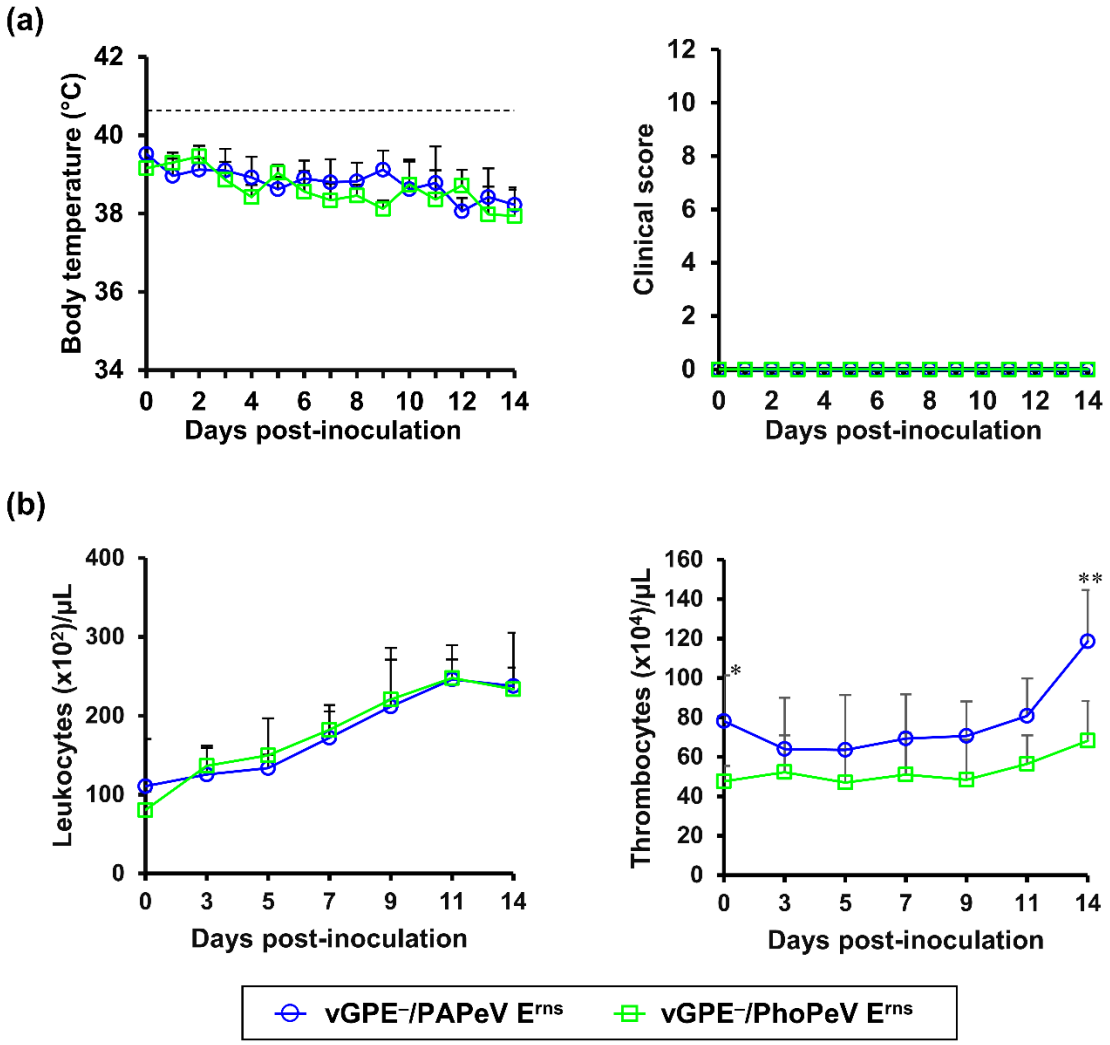


Figure 2. Body temperature, clinical score, leukocyte, and thrombocyte counts of pigs inoculated with the vGPE⁻/PAPeV E^{rns} or vGPE⁻/PhoPeV E^{rns}. Groups of 5 pigs were inoculated with each virus, and blood was collected on 0, 3, 5, 7, 9, 11, and 14 dpi. **(a)** The body temperature and clinical score were daily monitored. High fever was defined as a body temperature of ≥ 40.5 °C (dashed horizontal lines). **(b)** The leukocyte and thrombocyte counts were measured at each time point. All objects are shown as mean values, with error bars representing the SDs. The significance of differences was calculated by the Student's *t*-test. *$p < 0.05$; ** indicates $p < 0.01$ between the vaccinated group.

Table 1. Virus recovery from blood samples and neutralizing antibody in pigs inoculated with vGPE^{-}/PAPeV E^{rns} or vGPE^{-}/PhoPeV E^{rns}

Virus	Pig ID	Virus recovery at dpi (log_{10} $TCID_{50}$/mL)							SNT at dpi	
		0	3	5	7	9	11	14	0	14
vGPE^{-}/PAPeV E^{rns}	#293	–	–	–	–	–	–	–	<1	2
	#294	–	–	–	–	–	–	–	<1	8
	#295	–	–	–	–	–	–	–	<1	4
	#296	–	–	–	–	–	–	–	<1	2
	#297	–	–	–	–	–	–	–	<1	8
vGPE^{-}/PhoPeV E^{rns}	#374	–	–	–	–	–	–	–	<1	1
	#375	–	–	–	–	–	–	–	<1	2
	#376	–	–	–	–	–	–	–	<1	2
	#377	–	–	–	–	–	–	–	<1	2
	#378	–	–	–	–	–	–	–	<1	<1

–, not isolated in a 6-well plate.

Table 2. Virus recovery from organ samples in pigs inoculated with vGPE^{-}/PAPeV E^{rns} or vGPE^{-}/PhoPeV E^{rns} at 14 dpi

Virus	Pig ID	Virus recovery (log_{10} $TCID_{50}$/g)						
		Tonsil	Brain	Spleen	Kidney	AG	LN	Colon
vGPE^{-}/PAPeV E^{rns}	#293	–	–	–	–	–	–	–
	#294	–	–	–	–	–	–	–
	#295	–	–	–	–	–	–	–
	#296	–	–	–	–	–	–	–
	#297	–	–	–	–	–	–	–
vGPE^{-}/PhoPeV E^{rns}	#374	–	–	–	–	–	–	–
	#375	–	–	–	–	–	–	–
	#376	–	–	–	–	–	–	–
	#377	–	–	–	–	–	–	–
	#378	–	–	–	–	–	–	–

–, not isolated in a 6-well plate.

Infectivity and immune responses in chimeric virus-inoculated pigs

Three independent animal experiments were conducted to assess the optimal infectious dose and immune responses of chimeric virus-inoculated pigs, which would be beneficial for determining the vaccination dose in the challenge study. In each animal experiment, nine pigs were randomly divided into groups of three. Pigs were inoculated via an intramuscular route with a dose of $10^{3.0}$, $10^{4.0}$, or $10^{5.0}$ $TCID_{50}$ of each virus vGPE$^-$, vGPE$^-$/PAPeV E^{rns}, or vGPE$^-$/PhoPeV E^{rns}. As shown in Figure 3a, neutralizing antibodies were detected, which demonstrated a tendency to increase in most inoculated pigs from 14 dpi until the end of the experiment at 21 dpi with different immune response levels dependent on the inoculated doses, although viremia was not detected in pigs inoculated with each virus (Table 3). In Figure 3b, either vGPE$^-$/PAPeV E^{rns} or vGPE$^-$/PhoPeV E^{rns} elicited CSFV-specific neutralizing antibodies comparable to the vGPE$^-$ vaccine strain in pigs inoculated with $10^{5.0}$ $TCID_{50}$ at 14 dpi; however, the absence of the neutralizing antibody was confirmed in several pigs that were inoculated with $10^{3.0}$ or $10^{4.0}$ $TCID_{50}$ of each virus at this time point. Accidentally, one dead pig (#361) was found at 18 dpi without any clinical signs and no detection of virus recovery before death (Table 3). The cause of death was unknown, as there were no gross pathological lesions or virus recovery in organ samples. After 21 days of inoculation with vGPE$^-$, vGPE$^-$/PAPeV E^{rns}, or vGPE$^-$/PhoPeV E^{rns}, all pigs produced CSFV-specific neutralizing antibodies, but only those pigs inoculated with $10^{4.0}$ or $10^{5.0}$ $TCID_{50}$ (Figure 3b). Most pigs developed immune responses at 21 dpi by inoculation with $10^{3.0}$ $TCID_{50}$, except for one pig in each vaccination group of vGPE$^-$ or vGPE$^-$/PhoPeV E^{rns} and two vGPE$^-$/PAPeV E^{rns}-inoculated pigs. These results demonstrated that vGPE$^-$/PAPeV E^{rns} and vGPE$^-$/PhoPeV E^{rns} induced CSFV-specific neutralizing antibody responses in pigs comparable to the parental strain vGPE$^-$. Considering the vaccination dose in the subsequent challenge studies, $10^{4.0}$ $TCID_{50}$ would be preferable as an optimal infectious dose for the vaccination that induced neutralizing antibodies in all pigs at the end of the experiment.

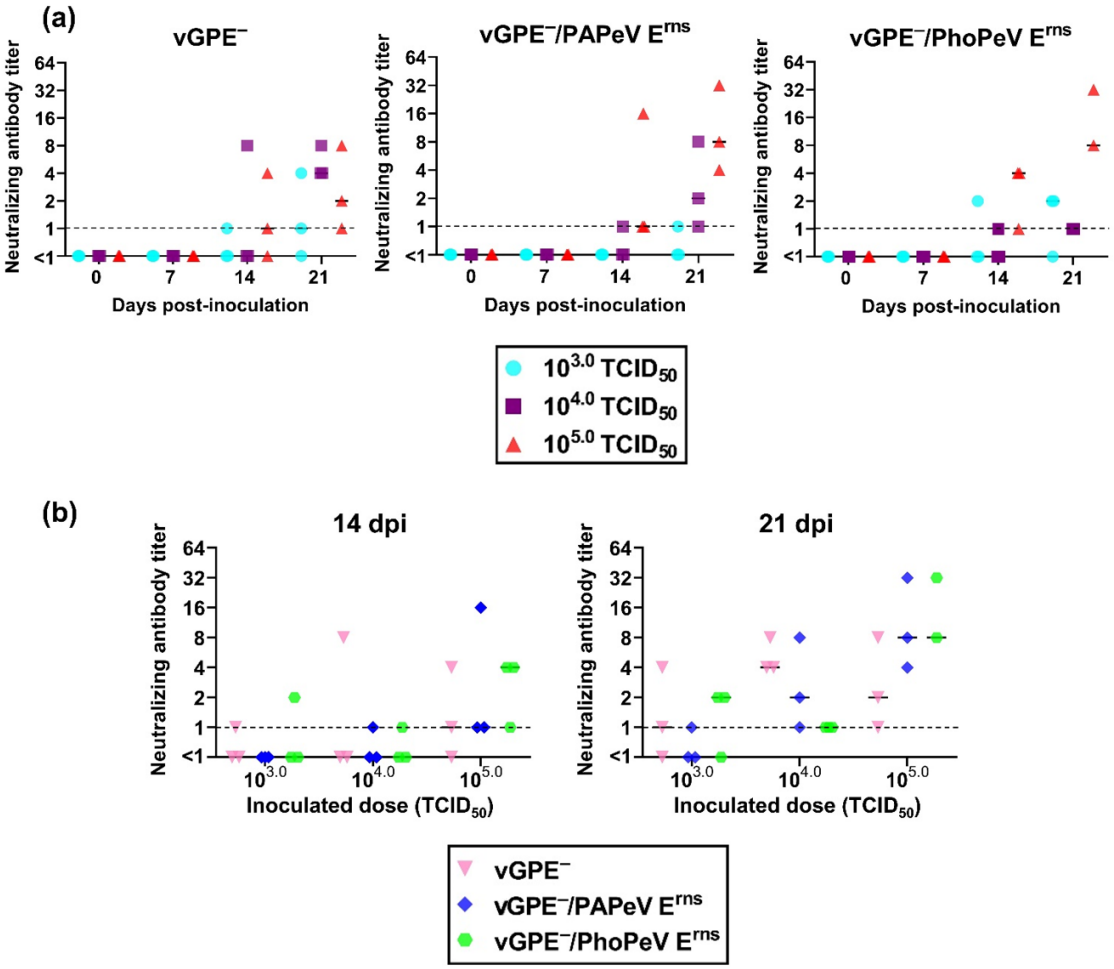


Figure 3. Neutralizing antibody titers in pigs inoculated with doses of $10^{3.0}$, $10^{4.0}$, and $10^{5.0}$ $TCID_{50}$ at 0, 7, 14, and 21 dpi. (**a**) Neutralizing antibody titers in pigs inoculated with vGPE⁻, vGPE⁻/PAPeV E^{rns}, or vGPE⁻/PhoPeV E^{rns}. (**b**) Neutralizing antibody titers at 14 dpi and 21 dpi in pigs inoculated with vGPE⁻, vGPE⁻/PAPeV E^{rns}, or vGPE⁻/PhoPeV E^{rns} at different doses of $TCID_{50}$. The dot-plot graph shows individual titers with the median in each group of $10^{3.0}$, $10^{4.0}$, and $10^{5.0}$ $TCID_{50}$ (n = 9). Dashed horizontal lines show the detection limit.

Table 3. Virus recovery from blood samples in pigs inoculated with vGPE$^-$, vGPE$^-$/PAPeV E^{rns}, or vGPE$^-$/PhoPeV E^{rns} at different doses of $TCID_{50}$

Virus	Inoculation dose ($TCID_{50}$)	Pig ID	Virus recovery at dpi (log_{10} $TCID_{50}$/mL) 0	7	14	21
vGPE$^-$/PAPeV E^{rns}	$10^{3.0}$	#312	–	–	–	–
		#313	–	–	–	–
		#314	–	–	–	–
	$10^{4.0}$	#315	–	–	–	–
		#316	–	–	–	–
		#317	–	–	–	–
	$10^{5.0}$	#318	–	–	–	–
		#319	–	–	–	–
		#320	–	–	–	–
vGPE$^-$/PhoPeV E^{rns}	$10^{3.0}$	#354	–	–	–	–
		#355	–	–	–	–
		#356	–	–	–	–
	$10^{4.0}$	#357	–	–	–	–
		#358	–	–	–	–
		#359	–	–	–	–
	$10^{5.0}$	#360	–	–	–	–
		#361	–	–	–	NS*
		#362	–	–	–	–
vGPE$^-$	$10^{3.0}$	#303	–	–	–	–
		#304	–	–	–	–
		#305	–	–	–	–
	$10^{4.0}$	#306	–	–	–	–
		#307	–	–	–	–
		#308	–	–	–	–
	$10^{5.0}$	#309	–	–	–	–
		#310	–	–	–	–
		#311	–	–	–	–

*no sample due to the pig being dead on 18 dpi; –, not isolated in a 6-well plate.

Efficacy of chimeric virus-vaccinated pigs against CSFV challenge

According to a clinical scoring system [48], there were no abnormalities in body temperature or clinical signs in all pigs until the challenge day in both trials (Figures 4 and 5). In the first trial, the individual body temperatures of vaccinated or control pigs were recorded (Figure 4a). After the challenge, pigs immunized with either vGPE^{-} or vGPE^{-}/PAPeV E^{rns} displayed no fever. In the control pigs, only one (#345) displayed fever (≥40.5 °C) at 12 dpc. There was also no typical sign in pigs vaccinated with vGPE^{-}/PAPeV E^{rns} (this phenomenon was also observed in pigs immunized with vGPE^{-}, although one pig (#349) developed mild clinical signs (hesitant walking or tiredness, getting up only when forced to and lying down again) from 6 to 12 dpc but finally recovered after that until 14 dpc. In contrast, control pigs developed moderately typical CSF symptoms (diarrhea, conjunctivitis, or low appetite) from 6 dpc until the end of the experiment (Figure 4b). Similar to the first trial, no fever was observed in pigs vaccinated with vGPE^{-}/PhoPeV E^{rns} after the challenge in the second trial (Figure 5a). Conversely, two control pigs (#368 and #370) had mild fever (40.3 °C) at 5 dpc which developed into acute fever (41.4 °C and 41.7 °C, respectively). Typical clinical signs were also found in control pigs in the second trial (Figure 5b), which developed mild clinical signs from 5 dpc and increased to moderate clinical signs until the end of the experiment, with the highest score of 10. Meanwhile, pigs vaccinated with vGPE^{-}/PhoPeV E^{rns} showed no typical clinical signs (Figure 5b).

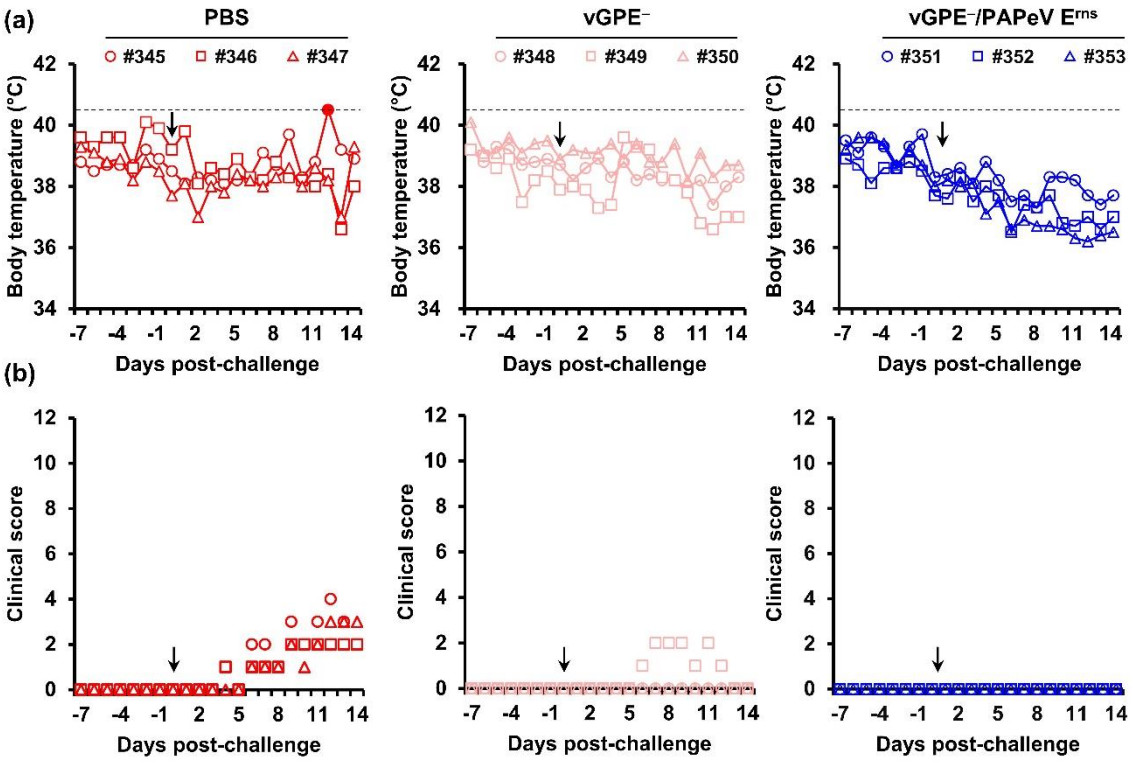


Figure 4. Body temperature and clinical scores of pigs in the first trial. Nine 2-week-old pigs were divided into three groups: PBS-vaccinated pigs (control) (red), vGPE^{-}-vaccinated pigs (pink), and vGPE^{-}/PAPeV E^{rns}-vaccinated pigs (blue). **(a)** Individual body temperatures of pigs in each group. High fever was defined as a body temperature ≥ 40.5 °C (dashed horizontal lines), as shown by the filled marker. **(b)** Clinical scores were monitored daily from –7 to 0 dpc until the end of the experiment. Clinical signs were scored on 10 parameters, ranging from 0 to 3 for each. The black arrow indicates the day of the virus challenge. All pigs were euthanized at 14 dpc.

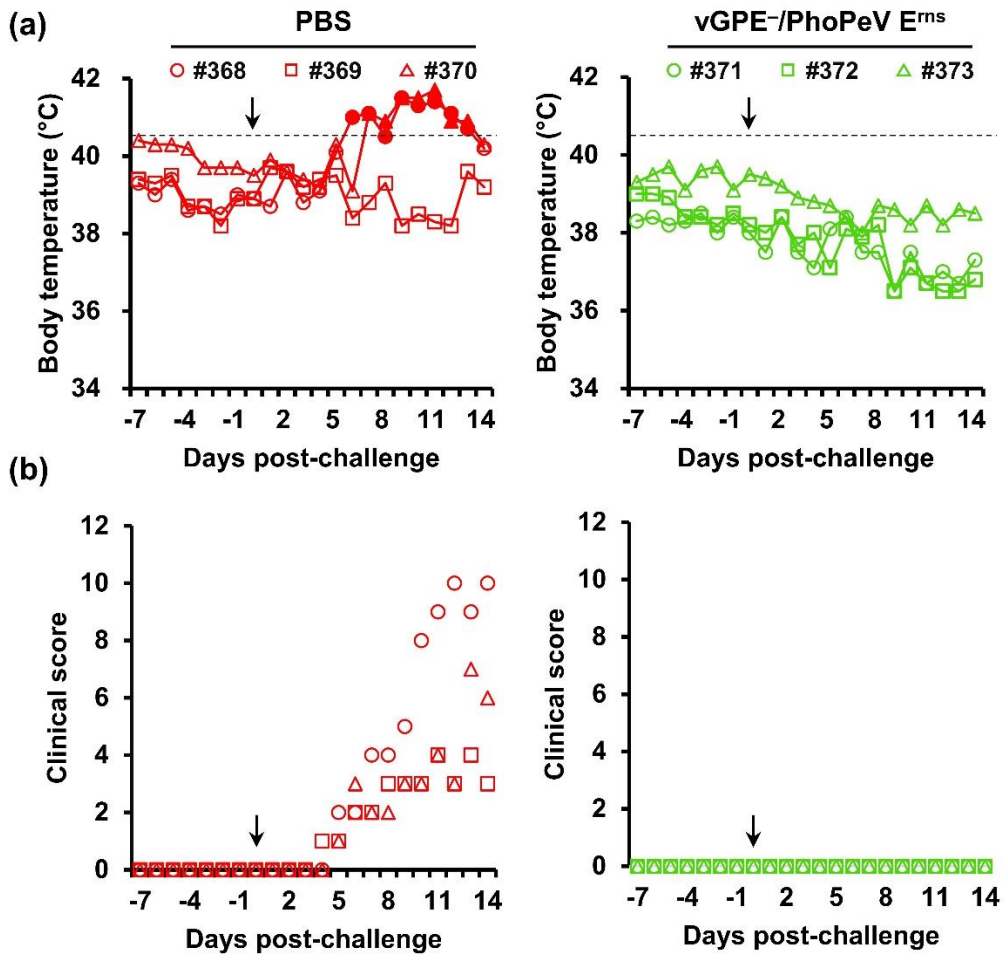


Figure 5. Body temperature and clinical scores of pigs in the second trial. Six 2-week-old pigs were divided into two groups: PBS-vaccinated pigs (control) (red) and vGPE^{-}/PhoPeV E^{rns}-vaccinated pigs (green). **(a)** Individual body temperatures of pigs in each group. High fever was defined as a body temperature ≥40.5 °C (dashed horizontal lines), as shown by the filled markers. **(b)** Clinical scores were monitored daily from –7 to 0 dpc until the end of the experiment. Clinical signs were scored on 10 parameters, ranging from 0 to 3 for each. The black arrow indicates the day of the virus challenge. All pigs were euthanized at 14 dpc.

Leukocyte and thrombocyte counts were utilized as an additional indicator to observe the disease manifestation of the CSFV challenge in pigs and evaluate the attenuation of chimeric viruses compared to the vGPE^{-} strain (Figure 6). Leukocyte and thrombocyte counts (Figure 6a) were dramatically decreased at 3 dpc in control pigs (93.3 ± 33.0 × 10^2 cells/μL and 35.1 ± 14.7 × 10^4 cells/μL, respectively) and remained at low concentrations (93.3 ± 33.0 × 10^2 cells/μL and 35.1 ± 14.7 × 10^4 cells/μL, respectively) until 14 dpc in the first trial. Meanwhile, in pigs immunized with either vGPE^{-} or vGPE^{-}/PAPeV E^{rns} there was a tendency for an increase, but with more variation, in the leucocyte and thrombocyte counts, which were the highest in vGPE^{-}-vaccinated pigs. Although slight transient thrombocytopenia was found in vGPE^{-}/PAPeV E^{rns}-vaccinated pigs at 3 dpc, recovery was observed later until 14 dpc. A similar tendency was found in the second trial (Figure 6b), in which vGPE^{-}/PhoPeV E^{rns}-vaccinated pigs showed an increase in leukocyte and thrombocyte counts from 0 dpc until the end of the experiment. In contrast, control pigs displayed a decrease in leukocyte counts and bottomed out at 9 dpc (38.3 ± 16.6 × 10^2 cells/μL), although recovery was observed after that. Thrombocytopenia in control pigs was also observed; however, it was not significantly different from that in the vaccinated group. Taken together, the increase in leukocytes and thrombocytes in vaccinated pigs in both trials showed vaccination-induced protective immune responses, whereas control pigs maintained the status of leukopenia and thrombocytopenia.

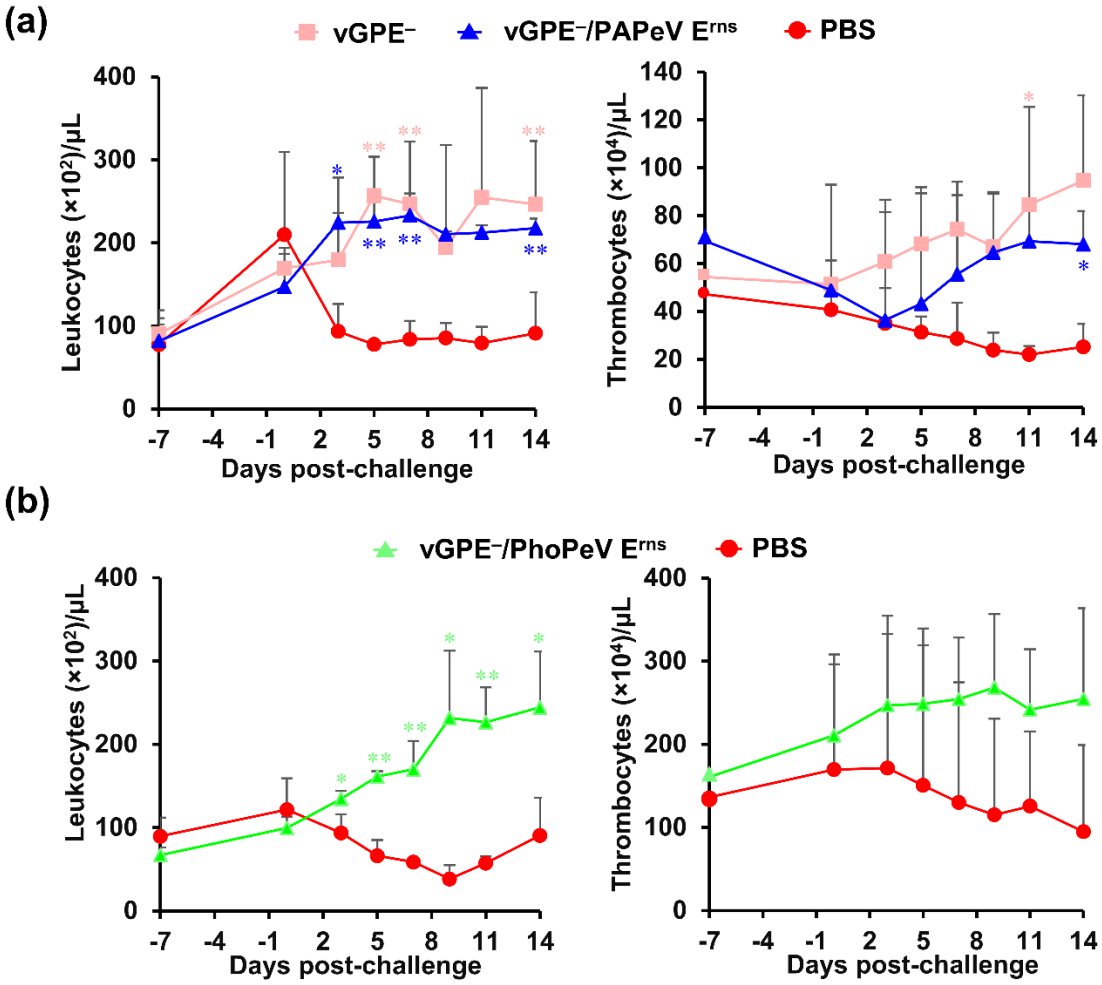


Figure 6. Leukocyte and thrombocyte counts of pigs in two challenge studies. (**a**) Pigs immunized with vGPE⁻ or vGPE⁻/PAPeV E^{rns} and PBS (control). (**b**) Pigs immunized with vGPE⁻/PhoPeV E^{rns} and PBS (control). Blood was collected to measure leukocyte and thrombocyte counts at each time point at −7, 0, 3, 5, 7, 9, 11, and 14 dpc. The data are shown as mean values, with error bars representing SDs. The significance of differences was calculated using one-way ANOVA, followed by the Student's *t*-test. * $p < 0.05$; ** indicates $p < 0.01$ between the vaccinated and control groups.

In both trials of the challenge study, the neutralizing antibody was under the detection limit in the control groups at 0 and 14 dpc (Figure 7a). In addition, the neutralizing antibody against vGPE^{-}/PAPeV E^{rns} or vGPE^{-}/PhoPeV E^{rns} was below the detection limit at 0 dpc. However, the neutralizing antibody was found in the vaccinated pigs at the end of the experiment (14 dpc), with the highest neutralizing antibody titer of 16 in vGPE^{-} or vGPE^{-}/PAPeV E^{rns}-vaccinated pigs, whereas pigs vaccinated with vGPE^{-}/PhoPeV E^{rns} showed the highest neutralizing antibody titer of 8.

Considering the role of cellular immunity in protecting pigs against CSFV challenge at an early stage of vaccination in the absence of neutralizing antibodies, IFN-γ induction in stimulated PBMCs was evaluated in all pigs at 0 dpc. As shown in the results (Figure 7b), pigs immunized with vGPE^{-}, vGPE^{-}/PAPeV E^{rns}, or vGPE^{-}/PhoPeV E^{rns} exhibited IFN-γ production in PBMCs stimulated with vGPE^{-}, whereas PBMCs from control pigs showed a deficient response to viral stimulation. In the first trial, there was no statistically significant difference between groups, although increased IFN-γ concentrations were detected in vGPE^{-} or vGPE^{-}/PAPeV E^{rns}-vaccinated pigs. Meanwhile, pigs immunized with vGPE^{-}/PhoPeV E^{rns} exhibited IFN-γ production at a high concentration, presenting a significant difference from control pigs in the second trial.

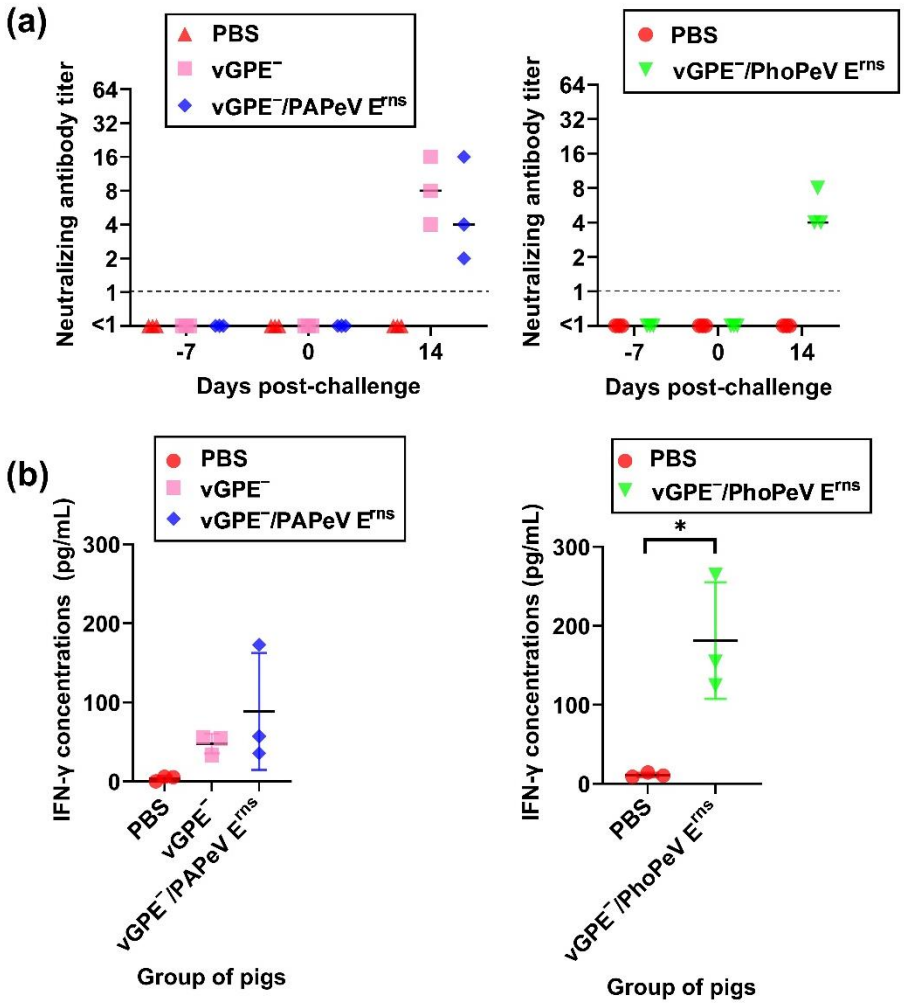


Figure 7. Neutralizing antibodies and cellular immune response in pigs after 7 days of vaccination. (**a**) Neutralizing antibodies in pigs vaccinated with vGPE$^-$, vGPE$^-$/PAPeV E^{rns}, or vGPE$^-$/PhoPeV E^{rns} and PBS (control). Dashed horizontal lines show the detection limit. (**b**) Detection of IFN-γ concentrations in PBMC culture supernatant by ELISA. The data are shown as mean values, with error bars representing SDs. The significance of differences was calculated using one-way ANOVA, followed by the Student's *t*-test. * $p < 0.05$, between the vaccinated and control groups.

Before the CSFV challenge, no virus was detected in pigs immunized with vGPE^{-}, vGPE^{-}/PAPeV E^{rns}, or vGPE^{-}/PhoPeV E^{rns} and control (Table 4). Notably, the absence of viral replication in the blood (Table 4) and organ (Table 5) samples in both vGPE^{-}/PAPeV E^{rns}-vaccinated and vGPE^{-}/PhoPeV E^{rns}-vaccinated pigs was evidenced by viral titration. A transient viremia was identified in one vGPE^{-}-vaccinated pig (#349) from 3 to 11 dpc (Table 4). At 14 dpc, virus recovery was confirmed in the tonsils and kidneys despite no viremia being detected in this pig (Table 5). However, the viral titer was under the detection limit of $10^{1.8}$ $TCID_{50}$/g.

In contrast, control pigs in both challenge trials showed increased viremia levels in blood samples starting from 3 dpc until the end of the experiment (Table 4). The virus recovery with high titers was confirmed in most control pigs. Virus isolation in organ samples was also conducted after the necropsy (Table 5). Only one pig (in the control group) showed a moderate virus titer in organ samples ($10^{2.0}$–$10^{4.0}$ $TCID_{50}$) compared to high titers of virus recovery (>$10^{4.0}$ $TCID_{50}$) in the other two pigs in the first trial. High virus titers (≥$10^{4.0}$ $TCID_{50}$) in organ samples were also confirmed in all control pigs in the second trial (Table 5). These results demonstrated that vaccination with either vGPE^{-}/PAPeV E^{rns} or vGPE^{-}/PhoPeV E^{rns} could protect pigs against systemic infection from CSFV challenge.

Table 4. Virus recovery from blood samples in pigs vaccinated with vGPE^{-}, vGPE^{-}/PAPeV E^{rns}, or vGPE^{-}/PhoPeV E^{rns} and PBS (control)

Trial	Virus	Pig ID	Virus recovery at dpc (log_{10} $TCID_{50}$/mL)							
			−7	**0**	**3**	**5**	**7**	**9**	**11**	**14**
First	vGPE^{-}/PAPeV E^{rns}	#351	–	–	–	–	–	–	–	–
		#352	–	–	–	–	–	–	–	–
		#353	–	–	–	–	–	–	–	–
	vGPE^{-}	#348	–	–	–	–	–	–	–	–
		#349	–	–	+	2.8	3.3	1.3	+	–
		#350	–	–	–	–	–	–	–	–
	Control	#345	–	–	1.8	3.6	4.6	6.0	6.1	6.8
		#346	–	–	1.5	2.8	3.6	3.8	3.3	2.8
		#347	–	–	1.3	3.6	4.3	6.3	5.8	6.0
Second	vGPE^{-}/PhoPeV E^{rns}	#371	–	–	–	–	–	–	–	–
		#372	–	–	–	–	–	–	–	–
		#373	–	–	–	–	–	–	–	–
	Control	#368	–	–	2.3	4.3	5.8	5.8	6.0	5.6
		#369	–	–	1.6	3.3	3.8	4.3	4.8	4.0
		#370	–	–	1.3	4.0	6.0	6.5	6.3	6.6

–, not isolated in a 6-well plate; +, isolated in a 6-well plate and lower than the detection limit of $TCID_{50}$ ($10^{0.8}$ $TCID_{50}$/mL) in a 96-well plate.

Table 5. Virus recovery from organ samples in pigs vaccinated with vGPE^{-}, vGPE^{-}/PAPeV E^{rns}, or vGPE^{-}/PhoPeV E^{rns} and PBS (control) at 14 dpc

Trial	Virus	Pig ID	Virus recovery (log_{10} $TCID_{50}$/g)						
			Tonsil	Brain	Spleen	Kidney	AG	LN	Colon
First	vGPE^{-}/PAPeV E^{rns}	#351	–	–	–	–	–	–	–
		#352	–	–	–	–	–	–	–
		#353	–	–	–	–	–	–	–
	vGPE^{-}	#348	–	–	–	–	–	–	–
		#349	+	–	–	+	–	–	–
		#350	–	–	–	–	–	–	–
	Control	#345	6.0	4.8	6.1	5.3	5.0	6.6	6.1
		#346	3.3	2.7	2.7	3.8	2.6	2.3	2.3
		#347	6.1	4.3	7.3	5.5	5.6	5.8	5.5
Second	vGPE^{-}/PhoPeV E^{rns}	#371	–	–	–	–	–	–	–
		#372	–	–	–	–	–	–	–
		#373	–	–	–	–	–	–	–
	Control	#368	5.0	4.0	6.0	5.8	5.8	5.0	5.0
		#369	4.6	4.0	4.8	5.0	4.8	5.0	4.8
		#370	6.3	4.3	6.6	6.8	6.8	5.8	5.0

–, not isolated in a 6-well plate; +, isolated in a 6-well plate and lower than the detection limit of $TCID_{50}$ ($10^{1.8}$ $TCID_{50}$/g) in a 96-well plate.

Discussion

Up to now, the design of MLVs for the CSF marker vaccine has principally focused on substituting the E^{rns} or E2 sequence [18,19,34,51,52], deletion of virulence-associated functional residues in E^{rns} combined with the substitution of the conformational epitope in E2 of CSFV [53], modifying E1 and E2 sequences or inserting the synthetic epitope on CSFV [54,55], and swapping the CSFV C-strain 5′-UTR, 3′-UTR, and partial E2 sequence with corresponding regions of BVDV [33]. Regarding the introduction of an exogenous sequence, chimeric vaccines based on the LAV strains offer a promising approach to taking advantage of the DIVA strategy by incorporating a specific marker into the vaccine strains [20,51]. Although a scheme for chimeric virus construction based on the genetic and antigenic distances of pestiviruses to CSFV has been developed based on the substitution of the CSFV E^{rns} glycoprotein with that of non-CSFV pestiviruses (NRPV, PAPeV, or a combination of both) to improve the DIVA strategy, which can diminish the possibility of inducing cross-reactive antibodies [19], the assessment of safe vaccines should be considered due to the use of the virulent CSFV strain as a backbone. Therefore, new chimeric viruses for CSF marker vaccine candidates were constructed in this study by substituting the full-length glycoprotein E^{rns} with that of PAPeV or PhoPeV based on the backbone of the Japanese GPE^{-} vaccine strain used in several countries and regions for decades [31,56].

In this study, an attempt to engineer GPE^{-} into a chimeric virus vaccine candidate based on the construction of distantly related genetic pestiviruses was successfully achieved. Although there were some concerns about the malfunctioning genome or poor replication of chimeric viruses possessing E^{rns} of distantly related pestiviruses reported in previous studies [19,57], the two chimeric viruses $vGPE^{-}$/PAPeV E^{rns} and $vGPE^{-}$/PhoPeV E^{rns} in this study showed high viral titers and genetic stability, which are comparable to the parental strain $vGPE^{-}$. A successful introduction of PAPeV E^{rns} into the CSFV Alfort-Tübingen backbone was previously achieved with a well-replicated chimeric virus or by swapping the E^{rns} sequence of giraffe, reindeer, or PAPeV with those of BVDV (National Animal Disease Laboratory strain), indicating that the CSFV backbone could bear a substitution of E^{rns} [19,57]. Interestingly, the chimeric virus containing E^{rns} of a novel PhoPeV in marine mammals [58], which is heterologous to viruses of terrestrial mammals, could be rescued and showed efficient replication *in vitro*

in this study. As these two chimeric viruses showed efficient replication *in vitro*, an investigation was conducted to assess their pathogenicity in pigs. However, the virus titer was below the detection limit in pigs, indicating that their replication was attenuated in pigs. As expected, two chimeric viruses demonstrated a safety characteristic similar to the vGPE$^-$ strain reported in a previous study [40].

The immunogenicity profiles of chimeric viruses were also evaluated in pigs inoculated with different doses that could be determined as optimal vaccination doses in the challenge study. Neutralizing antibodies against CSFV were detected in all inoculated pigs without the detection of viremia, except for several pigs inoculated with the lowest $TCID_{50}$ dose. Antibodies against the structural (E2, E^{rns}) and non-structural (NS3) proteins have been detected, with E2 being the most immunogenic and essential to induce the neutralizing antibody [9,59]. In line with previous studies [19,57], substituting E^{rns} in the vGPE$^-$ backbone with heterologous E^{rns} of non-CSFV pestiviruses in this study may exert less effect on the induction of neutralizing antibodies. In fact, the present experimental conditions in this study could not fulfill the vaccination guidelines and long-term observation period, which would provide enough time to induce antibody responses in pigs [38]. However, an increased level of neutralizing antibodies was observed in all inoculated pigs at all different doses of $TCID_{50}$ during the experiment, proving that both chimeric viruses exhibited comparable immunogenicity with the parental vGPE$^-$ strain.

Remarkably, this study proved its efficacy against moderately virulent CSFV challenges as early as 7 days after vaccination. In detail, pigs immunized with either the chimeric virus vGPE$^-$/PAPeV E^{rns} or vGPE$^-$/PhoPeV E^{rns} showed early protection against clinical signs and systemic viral replication. In contrast, control pigs exhibited fever and moderate clinical signs. The difference in the clinical score was observed in control pigs in both trials, which could be explained by the less severe clinical signs caused by the CSFV vALD-A76 challenge according to the clinical scoring system. Leukocytopenia and thrombocytopenia were observed along with long-lasting viremia by the CSFV challenge strain [42,60]. In this regard, the protective capability of chimeric viruses against CSFV systemic infection in pigs is shown to elicit an early-onset protective immune response against CSF. The advantage of a vGPE$^-$-based backbone, as proven in a previous study, is that it would promote a fitting backbone in developing the CSF marker vaccine [40]. It was previously shown that the LAV GPE$^-$ has been proven to

confer early protection even at 3 dpv against CSFV challenge to prevent the development of clinical signs and viral replication [61]. The protection conferred to vaccinated pigs at 7 dpv against the CSFV challenge in this study was not solely dependent on the antibody response because the neutralizing antibody titer was undetectable in all pigs on the day of the challenge. Despite the absence of an antibody response before the CSFV challenge, neutralizing antibody titers were detected at the end of the challenge study in all vaccinated pigs compared to the lack of them in control pigs. The increase in neutralizing antibodies in vaccinated pigs could be associated with clinical and virological protection. As the activation of cell-mediated immune (CMI) responses also contributed to early protection after the CSFV challenge in pigs [50,61,62], this study investigated whether the presence of CSFV-specific IFN-γ-secreting cells could be correlated with disease protection after 7 dpv. CMI response is vital in regulating the immune response and essential for providing immunity against intracellular pathogens. In addition, the level of antigen-specific IFN-γ production could be used as an indicator of CMI response [63]. Notably, IFN-γ production from *in vitro* stimulated PBMCs was detected at 7 dpv in vaccinated pigs, which could not be observed clearly in the control pigs. This finding was consistent with previous studies showing that IFN-γ production could be detected in 5-week-old crossbred pigs after 6 dpv with C-strain [63]. However, recent studies have indicated that the IFN-γ secretion from PBMCs was not detected in pigs at 5 dpv, and $CD4^+$ and $CD8^+$ T cells were detected as the cellular source of CSFV-specific IFN-γ-secreting cells that could be seen after the CSFV challenge day [50,62]. In this study, the presence of CSFV-specific IFN-γ-secreting cells seems to be correlated with early protection against systemic infection following the CSFV challenge by 7 days after vaccination. Despite IFN-γ production on the day of the challenge as shown in this study, the crucial role of CSFV-specific antibodies should not be excluded, as there was an increased level of neutralizing antibodies in vaccinated pigs but not in control pigs. IFN-γ detection is believed to be an alternative way to assess T cell response to CSFV; however, the T cell phenotypes in PBMCs, including IFN-γ-producing CD4 or CD8 T cells, were not characterized in this study. Therefore, further studies are needed to evaluate the role and the initial source of CSFV-specific IFN-γ-producing T cells after vaccination.

This study was conducted to design and evaluate chimeric viruses with the target of providing early-onset protection against CSFV challenge. As it is also essential to prepare a serum panel at different time points in a long-term period after vaccination for test validation of DIVA potential, the antibodies against E^{rns} glycoprotein throughout these experiments were still not evaluated because of the present experimental conditions. Therefore, to differentiate chimeric vaccinated pigs from those infected with the CSFV challenge, immunoassays or POC diagnostic tools based on CSFV E^{rns}, PAPeV E^{rns}, and PhoPeV E^{rns} antigens and a serum panel should be developed to serologically detect pigs vaccinated with these two chimeric viruses, which will fulfill the DIVA strategy in future work.

Brief summary

A previous study proved that vGPE$^-$ mainly maintains the properties of CSFV, which is comparable to the GPE$^-$ vaccine seed and is a potentially valuable backbone for developing a CSF marker vaccine. Chimeric viruses were constructed based on an infectious cDNA clone derived from the LAV GPE$^-$ strain as novel CSF vaccine candidates that potentially meet the DIVA concept by substituting the glycoprotein E^{rns} of the GPE$^-$ vaccine strain with the corresponding region of non-CSF pestiviruses, either PAPeV or PhoPeV. High viral growth and genetic stability after serial passages of the chimeric viruses, namely vGPE$^-$/PAPeV E^{rns} and vGPE$^-$/PhoPeV E^{rns}, were confirmed *in vitro*. *In vivo* investigation revealed that two chimeric viruses had comparable immunogenicity and safety profiles to the vGPE$^-$ vaccine strain. Vaccination at a dose of $10^{4.0}$ $TCID_{50}$ with either vGPE$^-$/PAPeV E^{rns} or vGPE$^-$/PhoPeV E^{rns} conferred complete protection for pigs against the CSF virus challenge in the early stage of immunization. Thus, the characteristics of vGPE$^-$/PAPeV E^{rns} and vGPE$^-$/PhoPeV E^{rns} affirmed their properties, as the vGPE$^-$ vaccine strain, positioning them as ideal candidates for future development of a CSF marker vaccine.

Chapter II

Development of a dual immunochromatographic test strip to detect E2 and E^{rns} antibodies against classical swine fever virus

Introduction

In CSF-endemic countries, strategies involving biological safety precautions and routine vaccination using traditional LAVs and subunit vaccines have been implemented to control and eradicate CSF [20,24]. Nevertheless, failure of vaccination programs is an essential factor that impedes CSF control due to immunosuppression in pig herds caused by persistent infection with moderately or low virulent CSFV strains and the interference between vaccine types and MDA in piglets [20]. These challenges contribute to reduced vaccine efficacy, resulting in sporadic outbreaks and CSF reemergence in vaccinated herds and leading to CSF-endemic disease [21,22,64]. Hence, with the enforcement of improved surveillance systems and the advancement of efficient vaccines, developing efficient antibody detection systems is crucial for enhanced CSFV control. Considering this scenario, a suitable serological antibody assessment through extensive screening is needed to monitor the vaccination immunity of pigs against CSFV in practice [65,66].

Currently, the evaluation of antibody responses against CSFV is performed by SNT and ELISA designed to detect antibody responses specific to the E2 or E^{rns} glycoprotein [23,24]. Notably, detecting E^{rns} antibodies is a reliable diagnostic test for DIVA using marker vaccines that induce a protective immune response specific to CSFV E2 while showing an absence of specific antibodies to CSFV E^{rns} [67–69]. Given this context, traditional immunoassays, such as SNT or ELISA, can validate DIVA properties and vaccination efficacy of several marker vaccine candidates that have been recently generated, including chimeric pestiviruses CP_E2alf [17] and Flc-LOM-BE^{rns} [18] or two novel chimeric viruses vGPE^{-}/PAPeV E^{rns} and vGPE^{-}/PhoPeV E^{rns} and E2 subunit vaccines [70,71]. However, these assays must be conducted in a laboratory setting and involve complex experimental procedures, significant time consumption, and substantial equipment and maintenance costs despite their high sensitivity and accuracy [2,23,24]. Therefore, a reliable and rapid DIVA assay should be considered to successfully implement marker vaccines and validate vaccination efficacy.

An ICS is the most rapid and reliable POC diagnostic tool for prompt policy regarding effective disease control strategies [25]. ICS provides benefits compared to traditional immunoassays, such as affordability, rapidness, user-friendliness, and high specificity and sensitivity [23,25]. Previously, an ICS for CSFV antibody detection was

developed based on the chimeric protein CnC2, generated from a specific region encoding E^{rns} (amino acids 109–160; assigned as Cn) and E2 (amino acids 1–176; assigned as C2) antigen epitopes of CSFV by the *E. coli* expression system [72]. However, protein expression by the *E. coli* system can lead to protein misfolding, which may reduce antibody detection efficacy [73]. Several ICS studies have been performed to improve CSFV E2 antibody detection by enhancing the protein expression system, such as the baculovirus expression system [73] or transgenic rice endosperm [74]. However, ICS for CSFV E2 antibody detection can only be used to monitor vaccination efficacy and early detection of antibody responses, whereas the DIVA test for detecting E^{rns} antibody is lacking.

Plant-based production systems are promising platforms for recombinant protein production due to their numerous advantages over traditional expression systems [75,76]. Among these plant systems, *Nicotiana benthamiana* (*N. benthamiana*), a close relative of tobacco, has gained widespread attention for its exceptional suitability for recombinant protein expression. *N. benthamiana* offers several advantages for recombinant protein production [77–79] and is fast-growing, easily cultivated, and amenable to genetic manipulation, making it an attractive host for protein expression studies. Furthermore, *N. benthamiana* also possesses a robust protein folding and posttranslational modification machinery, allowing for the production of complex, biologically active proteins. A key advantage of *N. benthamiana* is its ability to produce glycosylated proteins with mammalian-like glycosylation patterns which is particularly advantageous for producing therapeutic proteins because proper glycosylation is often critical for protein stability, functionality, and immunogenicity [80]. Therefore, *N. benthamiana*-based expression system highlight their advantages, applications, and prospects.

As mentioned previously, two chimeric pestiviruses, namely vGPE^{-}/PAPeV E^{rns} and vGPE^{-}/PhoPeV E^{rns}, were generated based on the backbone of a CSFV LAV strain combined with the glycoprotein E^{rns} distantly related to CSFV. These two chimeric viruses were proposed as promising marker vaccine candidates, suggesting a feasible approach for establishing an enhanced serological negative marker in DIVA vaccine development for CSF. However, DIVA capability was still not assessed for these chimeric vaccine candidates.

Thus, in this study, a novel ICS was developed to rapidly and reliably detect CSFV E2 and E^{rns} antibodies. Recombinant E2 and E^{rns} proteins were transiently expressed in *N. benthamiana* using *Agrobacterium tumefaciens* (*A. tumefaciens*). Subsequently, ICS was constructed to detect CSFV E2 and E^{rns} antibodies. In addition, ICS performance was compared to commercial ELISA kits for CSFV E2 or E^{rns} antibody detection. Furthermore, a panel of serum samples derived from pigs vaccinated with chimeric vaccine candidates was utilized, incorporating ICS to evaluate their DIVA capability.

Materials and Methods

Cloning of CSFV E2 and E^{rns} genes

The full-length E2 (derived from CSFV Alfort A19 strain) and E^{rns} (derived from LOM strain) encoding sequences were obtained from the National Center for Biotechnology Information (NCBI) database (GenBank accession numbers AAB50409.1 and ABD83960, respectively) and optimized for expression in *N. benthamiana* using codon optimization program (https://zendto.bioneer.co.kr/codon/index.py) before gene synthesis. For CSFV E2 expression, genes encoding the signal peptide of binding immunoglobulin protein with a length of 271 nucleotides, the endoplasmic reticulum (ER) chaperone, full-length CSFV E2 sequence (1,023 nucleotides), linker (CAGATTACGACATTCCAACAACTGATGCA), 6 histidines, cellulose binding domain (454 nucleotides) derived from xylanase A of *Clostridium stercorarium*, and ER retention signal (His-Asp-Glu-Leu) were fused sequentially and then cloned into the pCAMBIA1300 vector [81] harboring the 35S promoter and heat shock protein terminator. The expected size of the fusion protein was approximately 58 kDa. For E^{rns} expression, genes encoding the signal peptide of chaperone binding protein (251 nucleotides), full-length CSFV E^{rns} (681 nucleotides), linker (GGTGGGGGAGGCAGT), 10 histidines, and ER retention signal (His-Asp-Glu-Leu) were fused sequentially and cloned into a pTEX vector harboring the MacT promoter and RD29B terminator. The expected size of the fusion protein was nearly 27.7 kDa. The nucleotide sequence was verified by sequencing (Bioneer, Daejeon, Republic of Korea).

Transient expression of recombinant CSFV E2 and E^{rns}

The plasmid for expressing recombinant CSFV E2 or E^{rns} was transformed into the *A. tumefaciens* GV3101 strain (Lifeasible, Shirley, NY, United States) by electroporation. Transformed *A. tumefaciens* were grown for 16 h in a 5 mL yeast extract peptone (YEP) liquid medium supplemented with 50 mg/L kanamycin and 25 mg/L rifampicin. Next, 1 mL of cultured bacteria was inoculated into 1 L of fresh YEP medium and cultured for a further 16 h at 28°C. Bacteria were pelleted by centrifugation at 7,341 × *g* for 5 min at 4°C and resuspended at the desired concentration (determined by measuring OD_{600}) in a solution consisting of 10 mM 2-(N-morpholino)ethane sulfonic acid (MES) (Duksan, Seoul, Republic of Korea), 10 mM magnesium chloride (Sigma-

Aldrich, St. Louis, MO, United States), and 100 μM acetosyringone (Sigma-Aldrich) at pH 5.6. Agroinfiltration was performed under a vacuum. After 4 days, the leaves were harvested for protein purification.

Purification of CSFV E2 and E^{rns} protein

In the purification of E2 protein, the leaves were crushed and incubated for 30 min in an extraction buffer (EB) 1 consisting of 50 mM sodium phosphate (Na_3PO_4; pH 8.0), 300 mM sodium chloride (NaCl), 10 mM imidazole, 0.5% (v/v) Triton X-100, 50 mM glycine, 100 mM sodium sulfite, 50 mM ascorbic acid, and 1.5% polyvinylpolypyrrolidone. With the purification of E^{rns} protein, an EB 2 consisting of 50 mM Tris-Cl (pH 8.5), 300 mM NaCl, 50 mM imidazole, 0.3% (v/v) Triton X-100, 100 mM glycine, 100 mM sodium sulfite, 1 mM phenyl methyl sulfonyl fluoride, and 1.5% polyvinylpolypyrrolidone was used. After centrifugation, the supernatant from EB 1 or EB 2 was mixed with His60 Ni Superflow Resin (Takara, Kusatsu, Japan) for 1 h at 4°C, and the bound proteins were eluted with 50 mM Na_3PO_4, 300 mM NaCl, and 250 mM imidazole for E2 protein or 300 mM imidazole for E^{rns} protein purification. The eluates were adjusted with 50 mM Tris-Cl (pH 7.2) and 150 mM NaCl. The purified proteins were treated with β-mercaptoethanol, assessed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and analyzed by Western blotting with an anti-His antibody (BioLegend, San Diego, CA, United States), anti-LOM vaccinated serum, and anti-E2 subunit vaccinated serum.

Manufacture of the prototype ICS

Colloidal gold solution preparation

The colloidal gold solution (in-house) was prepared by adding 90 mL of distilled water into a triangular flask and heating it to 100°C while stirring on a hot stirrer plate (DAIHAN Scientific, Wonju, Republic of Korea). Once the temperature reached 100°C, 1% (w/v) gold chloride trihydrate (10 mL) was added and dissolved. After the gold chloride trihydrate solution was completely dissolved, 1 mL of 1% sodium citrate tribasic dehydrate was added and dissolved for 10 min at room temperature until the color of the reaction solution changed from yellow to the final red color. The resulting colloidal gold

solution was diluted with distilled water, and the absorbance was measured using a UV-1650PC spectrophotometer (Shimazu, Tokyo, Japan).

Conjugation of the CSFV E2 and E^{rns} and pretreatment of the conjugate pad

For the conjugCoation of recombinant proteins to the colloidal gold, 50 mL of colloidal gold solution was adjusted to pH 7.2 using 0.1 molar potassium carbonate. Next, the recombinant E2 or E^{rns} solution with a concentration of 50 μg/mL in PBS was slowly added dropwise, one drop at a time, into the colloidal gold solution, and the mixture was stirred for 30 min on a stirrer. Then, 10% (w/v) bovine serum albumin (BSA; 5 mL) was added and stirred for an additional 30 min. The resulting conjugation solution was centrifuged to separate the upper layer for 30 min at 10,000 rpm, and the pellet was suspended in a solution containing 1% (w/v) BSA in PBS. Subsequently, to pretreat the conjugate pad with glass fiber material, a solution containing PBS, 3% sucrose, 0.1% sodium azide, 1% (w/v) BSA, recombinant E2 or E^{rns} conjugate (1.1 μg/kit), and rabbit IgG (in-house) conjugate (0.88 μg/kit), was evenly absorbed into the glass fiber material of the conjugate pad. The conjugate pad was dried for >4 h in an environment with a relative humidity of <20%.

Fixation of the control and test lines and kit assembly

The control and test lines were fixed using nitrocellulose membranes (Adventec, Irvine, CA, United States). The control line was coated with 1 mg/mL goat anti-rabbit IgG (Arista Biologicals, Allentown, PA, United States), and the test line was coated with each recombinant protein (E2 or E^{rns}) at 1 mg/mL. The membranes were dispensed using a dispenser, Matrix 1600 (Kinematic Automation, Sonora, CA, United States), at a rate of 1 μL/cm and dried for >4 h in an environment with a relative humidity of <20%. In the next step, the kit was assembled by attaching the membrane coated with the antigen onto the adhesive card, followed by the conjugate pad and sample pad overlaid to overlap at the bottom. The assembled kit was cut to a width of 4 mm and inserted into a plastic device.

Swine serum samples

Samples for evaluating the sensitivity and specificity of ICS

ICS sensitivity was assessed using 23 serum samples with varying SNT titers obtained from a previous study [82], sourced from two distinct groups: LOM-vaccinated pigs (n = 10) and nonvaccinated pigs with MDA (n = 13). Furthermore, ICS specificity was evaluated using swine serum samples (n = 5) that were positive for antibodies against CSFV, BVDV, or BDV from a previous study [83]. Additionally, CSFV antibody-negative serum samples (n = 50) from naïve pigs were included in the specificity analysis. Furthermore, the serum samples (n = 9) were obtained from pigs infected with African swine fever virus (ASFV), porcine circovirus 2 (PCV2), and porcine reproductive and respiratory syndrome virus (PRRSV) that were confirmed positive using commercial ELISA kits: ID Screen® African Swine Fever Indirect (Innovative Diagnostics, Grabels, France), PCV2 Antibody Test Kit (BioChek, Scarborough, ME, United States), and IDEXX PRRS X3 Ab Test (IDEXX, Westbrook, ME, United States), respectively.

Sera for determining the coincidence rates between the ICS and commercial ELISA kits

Six CSFV-negative piglets were purchased from a CSFV-negative farm located in Jeju Island, Republic of Korea, and confirmed by screening with an ELISA assay for E2 antibody detection. Piglets were injected intramuscularly twice on the neck at 40 days of age (0 dpv) and 60 days (20 dpv) after birth using the CSFV E2 subunit vaccine (BioApplications, Pohang, Republic of Korea), which was developed and evaluated in previous studies (Park et al., 2020, 2021). Sera were collected at 40 days of age (before vaccination), 60 days (20 days after the first vaccination), and 80 days (40 days after the first vaccination). Eighteen serum samples were used to compare the agreement rate between ICS and ELISA kits.

Recently, the LOM strain initially employed as a vaccine strain, was introduced to Jeju Island, where is known for its no-vaccination policy against CSFV. Unfortunately, the CSFV has started spreading from sows to piglets and has undergone mutations during its transmission. Consequently, the Government of the Republic of Korea is promoting a CSFV marker vaccine policy to combat and eliminate CSF [82]. To gather clinical sera for analysis, 57 serum samples were randomly obtained from CSFV-positive and -negative farms, subsequently determined to be positive and negative for E2 and E^{rns} antibodies by ELISA kits, respectively. These samples were used to evaluate the coincidence rates of ICS with ELISA kits for detecting E^{rns} and E2 antibodies.

The definition and way of calculation of total coincidence rates and positive coincidence rates are as followed [85]: total coincidence rates = 100% × [(both positive of ICS and ELISA kit + both negative of ICS and ELISA kit)/total samples]; compared to ELISA kit, the positive coincidence rates of ICS = 100% × [both positive of ICS and ELISA kit/(both positive of ICS and ELISA kit + ICS positive but ELISA kit negative)].

The performance of ICS was compared with commercial ELISA kits in terms of relative sensitivity and specificity, as followed [86]: relative sensitivity = 100% × [(the number of true positive samples/(the number of true positive samples + the number of false negative samples)]; relative specificity = 100% × [(the number of true negative samples/(the number of true negative samples + the number of false positive samples)].

Evaluation of the ICS using serum samples from pigs vaccinated with chimeric virus and commercial LAV

Serum samples of pigs vaccinated with novel chimeric vaccine candidates obtained from Chapter I, were used to evaluate DIVA properties using a novel ICS. In detail, 52 serum samples from twenty-six pigs vaccinated with each chimeric virus candidate (vGPE^{-}/PAPeV E^{rns} or vGPE^{-}/PhoPeV E^{rns}) or vGPE^{-} derived from a conventional LAV were evaluated at 0 and 21 dpv. Additionally, 45 serum samples from fifteen pigs were collected at 0, 7 dpv and 14 dpc in challenge studies that were also assessed. In these trials, each chimeric virus candidate or vGPE^{-} was independently administered to pigs 7 days before exposure to the moderately virulent CSFV vALD-A76 strain. Subsequently, continuous monitoring was conducted for 14 days. Furthermore, the vaccination efficacy of piglets with MDA (n = 6) immunized with a commercial conventional CSFV GPE^{-} LAV strain was also investigated, involving the collection of 24 serum samples at different time intervals (0, 45, and 90 dpv) and before slaughtering.

Enzyme-linked immunosorbent assay

The assay was performed using the PrioCHECK CSFV E^{rns} Antibody ELISA Kit (Thermo Fisher Scientific, Waltham, MA, United States), CSFV Antibody Test Kit (IDEXX, Westbrook, ME, United States), CSFV Antibody B-ELISA (Bionote, Gyenggi-do, Republic of Korea), and Pigtype CSFV E^{rns} Antibody (Indical Bioscience, Leipzig, Germany). The assay procedures were performed according to the manufacturers'

protocol. The absorbance of the solution was subsequently read at 450 nm on a Muliskan ELISA reader (Thermo Fisher Scientific).

Serum neutralization test

The determination of neutralizing antibody titers in serum samples derived from pigs vaccinated with each chimeric virus candidate, vGPE$^-$, or commercial conventional CSFV LAV GPE$^-$ strain was accomplished through a luciferase-based SNT, as outlined previously [49] and described thoroughly in Chapter I.

The neutralizing antibodies against CSFV in serum samples from a previous study [82] were tested using a neutralizing peroxidase-linked assay described previously [84]. In brief, equal volumes of serially diluted serum and 200 $TCID_{50}$ of the CSFV LAV LOM strain were mixed well and incubated at 37°C in 5% CO_2 for 1 h. The mixture and 100 µL cloned porcine kidney cell suspension were incubated in 96-well plates at 37°C in 5% CO_2 for 72 h. Cells were fixed with 80% acetone and dried at 37°C, followed by staining with commercial anti-LOM antibody as the primary antibody for IPX [84]. The SNT titers represent the reciprocal of the highest dilution that achieved 50% neutralization.

Ethics statement

The serum samples were subjected to evaluation of the specificity and DIVA diagnostic capability of the ICS, presented in Tables 7, 11, 12 and 13, originated from the animal experiments that were authorized by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine, Hokkaido University (approval no. 18-0038, approved on March 26, 2018, and approval no. 23-0029, approved on March 23, 2023) and performed according to the guidelines of this committee. The genetic modification experiments were performed according to the Cartagena Protocol on Biosafety under the Convention on Biological Diversity and approved by the Institutional Committee on the Safety of Genetic Modification Experiments of Hokkaido University and the Ministry of Education, Culture, Sports, Science and Technology (approval no. 2020-009 and 5-Monkashin-Dai-479, approved on June 8, 2021 and August 31, 2023, respectively).

Results

Expression and purification of recombinant CSFV E2 and E^{rns} proteins

The sequences encoding CSFV E2 and E^{rns} proteins were cloned into the *A. tumefaciens* GV3101 strain, and either the E2 or E^{rns} recombinant proteins were expressed in *N. benthamiana* plants. The harvested leaves were crushed and subjected to His60 Ni Superflow Resin for protein purification. The purified proteins were then assessed by Western blotting and SDS-PAGE. As illustrated in Figure 8, the glycosylated forms of recombinant E2 and E^{rns} proteins were confirmed by anti-His antibody. The recombinant E2 protein exhibited a visible band of ~69 kDa, whereas the recombinant E^{rns} protein appeared at ~45 kDa under denaturing conditions (Figure 8). Moreover, verification of the aforementioned findings was achieved by anti-LOM vaccinated serum (demonstrating positivity for E2 and E^{rns}) and anti-E2 subunit vaccinated serum (displaying positivity exclusively for E2) for the purpose of validating protein expression via Western blot analysis (Figure 9). Although the bands appeared to be diffuse in the analysis of E^{rns} protein, the deglycosylation with glycosidase PNGase F resulted in a single intense band, and the molecular weights of E^{rns} were approximately 28 kDa, which corresponded to its calculated molecular weights (Figure 9). Moreover, the intense band exceeded the anticipated molecular weights of recombinant E2 (58 kDa) or E^{rns} (28 kDa) fusion protein, suggesting that two proteins were extensively glycosylated, particularly with *N*-glycosylation contributing to sizes larger than anticipated that were observed when expressed in plants [81,84]. Taken together, the different glycosylation levels of each protein indicate that these two proteins are properly glycosylated and can serve as suitable antigens for further experiments.

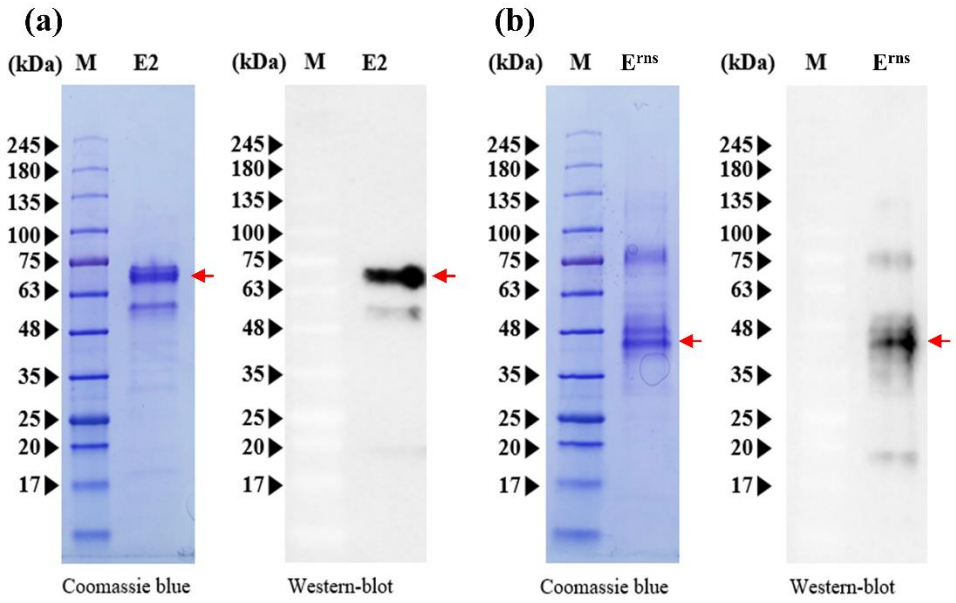


Figure 8. Expression and characterization of CSFV E2 and E^{rns} proteins. **(a)** Purified E2 protein was subjected to Western blotting (right panels) using an anti-His mAb, and gels were stained with Coomassie brilliant blue (left panels) after SDS-PAGE analysis. **(b)** Western blot analysis of purified E^{rns} protein (right panels) using an anti-His mAb and SDS-PAGE analysis (left panels). M, protein molecular weight marker. Red arrows represent the expression of E2 or E^{rns} protein.

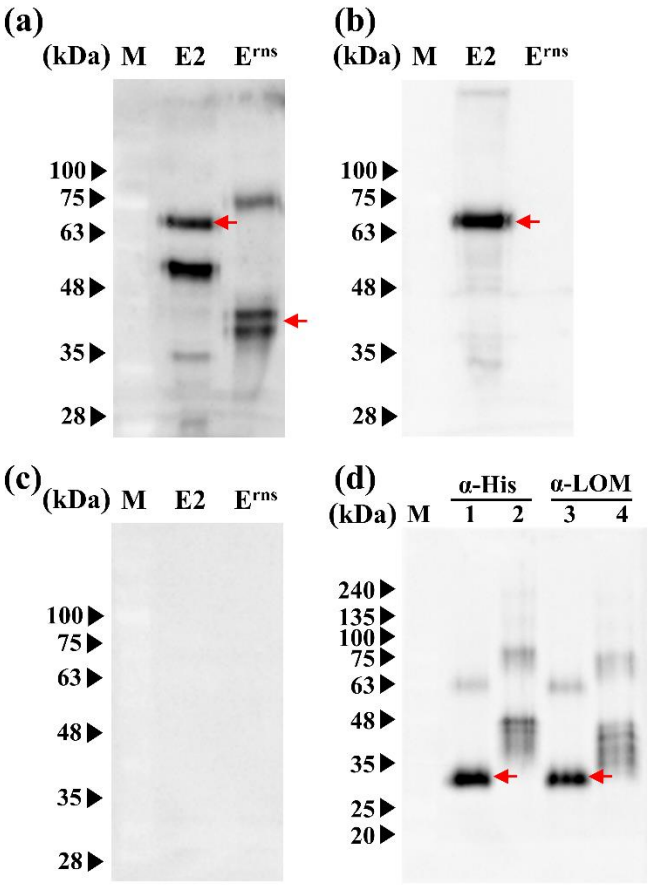
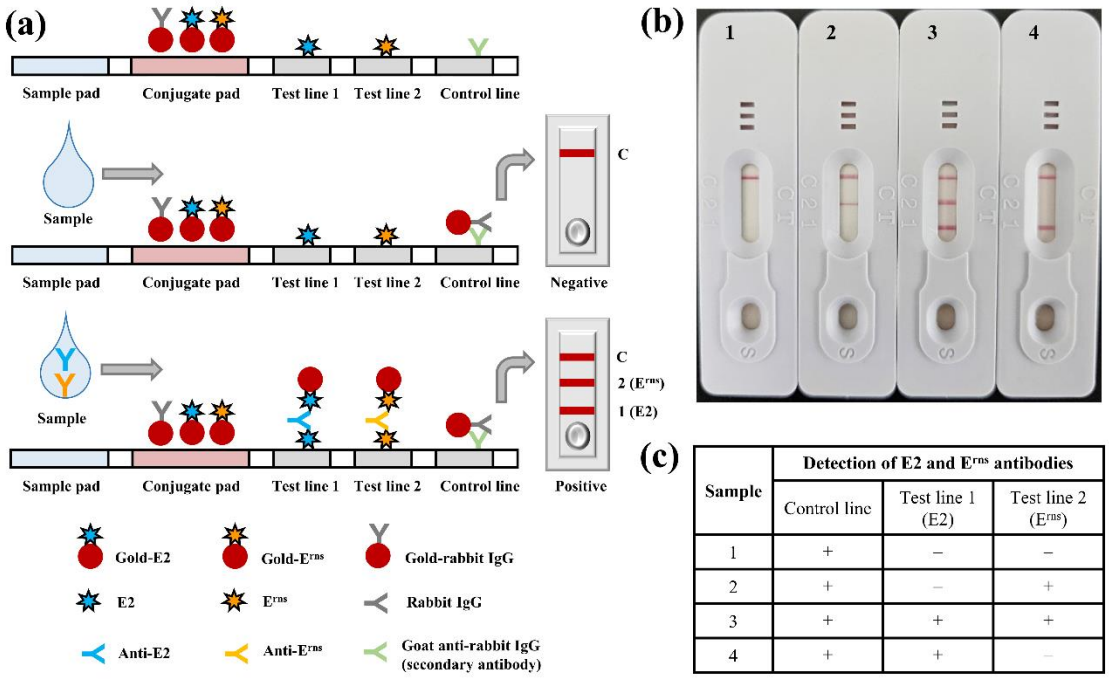


Figure 9. Expression and identification of CSFV E2 or E^{rns} protein. **(a)** Western blot analysis confirmed the recombinant CSFV E2 or E^{rns} protein using anti-LOM vaccinated serum. **(b)** The recombinant CSFV E2 or E^{rns} protein was confirmed in Western blotting using anti-E2 subunit vaccinated serum. **(c)** Western blotting using anti-native porcine serum as negative control. **(d)** Western blot analysis of purified E^{rns} protein using anti-His antibody for lane 1 (treated with PNGase F), and lane 2 (without PNGase F), or using anti-LOM vaccinated serum for lane 3 (treated with PNGase F) and lane 4 (without PNGase F). M, protein molecular weight marker. Red arrows represent the expression of E2 or E^{rns} protein.

Conjugation of recombinant proteins with the gold nanoparticles and implementation of the working principle of the developed CSFV E2 and E^{rns} detection kit

The optimal protein concentration for creating the E2 or E^{rns} conjugate was 1.25 OD, using a spectrophotometer at a wavelength of 540 nm and a particle size of 40 nm. The ICS assembly is illustrated in Figure 10. The assembled ICS comprised a membrane coated with the antigen onto the adhesive card, followed by a conjugate pad and sample pad overlaid to overlap at the bottom. Nitrocellulose membranes were used to fix the control line (C) and test line (T) membranes in the ICS. Specifically, the control line was plated with a second antibody (goat anti-rabbit IgG). Meanwhile, the test line was plated with recombinant E2 and E^{rns} proteins corresponding to numbers 1 and 2 in the ICS (Figure 10a). In accordance with usage principles, serum samples from pigs were loaded on the application pad and diffused with gold-rabbit IgG and gold-E2 or E^{rns} on the conjugate pad. For the control line, gold-rabbit IgG is bound to anti-rabbit IgG antibodies and accumulated. The anti-E2 or E^{rns} antibody in the serum sample is bound and diffused with gold-E2 or E^{rns} on the conjugate pad, and the complex bound to the E2 or E^{rns} protein on the test line. Consequently, a red color appeared on the test line for the positive samples due to the accumulated complex. In contrast, the test line will not display red color for the negative samples that did not have anti-E2 or E^{rns} antibodies (Figures 10b,c).



Sample	Detection of E2 and E^{rns} antibodies		
	Control line	Test line 1 (E2)	Test line 2 (E^{rns})
1	+	–	–
2	+	–	+
3	+	+	+
4	+	+	–

Figure 10. Diagrammatic illustration and implementation example of the ICS. **(a)** Structural composition of the ICS, including sample and conjugate pads, test lines 1 and 2, and control line; negative and positive results were also indicated. **(b)** The implementation of representative serum samples using the ICS 1 (negative control), 2 (positive with E^{rns}), 3 (positive with both E2 and E^{rns}), and 4 (positive with E2), in which the serum samples were diluted with dilution buffer at a ratio of 1:1. **(c)** Representative results of each sample were summarized in the table.

Specificity and sensitivity of the ICS

The sensitivity of ICS was evaluated using various serum samples positive for CSFV with different SNT titers. Additionally, the specificity of ICS was assessed using sera from CSFV- and pestivirus-inoculated pigs, and sera from naïve pigs. Twenty-three serum samples ranging from the lowest SNT titer of 4 to the highest SNT titer of 2,048 were subjected to ICS testing. The results in Table 6 demonstrated that ICS effectively detected antibodies against CSFV E2 and E^{rns} in all samples. The E2 and E^{rns} antibodies were detected at the lowest SNT titers of 16 and 4, respectively. These findings demonstrated the high sensitivity of the ICS for detecting E2 and E^{rns} antibodies, with particular sensitivity for CSFV E^{rns} antibody detection. Furthermore, the specificity of ICS for CSFV E2 and E^{rns} antibody detection was assessed. Table 7 revealed that two CSFV-positive serum samples were also confirmed for detecting both E2 and E^{rns} antibodies using ICS. In contrast, other pestivirus-positive serum samples (n = 3), including BVDV strains Nose (BVDV genotype 1a) and KZ-91-NCP (BVDV genotype 2a), BDV, and CSFV-negative sera from naïve pigs (n = 50) tested negative for E2 and E^{rns} antibodies. Furthermore, additional serum samples (n = 9), consisting of ASFV (n = 3), PCV2 (n = 3), and PRRSV (n = 3) were also confirmed as negative. Data suggested that the ICS exhibited no cross-reactivity with antibodies from other swine pestiviruses and related swine viral diseases.

Table 6. Sensitivity of the ICS for detecting E2 and E^{rns} antibodies using various swine serum samples with different neutralizing antibody titers

Neutralizing antibody titers	Antibody detection by ICS				Total
	E2		E^{rns}		
	Positive	Negative	Positive	Negative	
4	0	1	1	0	1
16	1	0	1	0	1
32	7	0	7	0	7
64	3	0	3	0	3
128	2	0	2	0	2
256	3	0	3	0	3
512	2	0	2	0	2
1,024	2	0	2	0	2
2,048	2	0	2	0	2

Table 7. Specificity of the ICS for detecting E2 and E^{rns} antibodies in reference swine serum samples

Serum samples	Serum at dpi	Antibody detection				Total
		E2		E^{rns}		
		Positive	Negative	Positive	Negative	
CSFV GPE^{-} positive*	33	1	0	1	0	1
CSFV Kanagawa/74- positive*	32	1	0	1	0	1
BVDV Nose-positive*	32	0	1	0	1	1
BVDV KZ-91 NCP-positive*	33	0	1	0	1	1
BDV 87/6-positive*	32	0	1	0	1	1
CSFV-negative	0	0	50	0	50	50
ASFV	7	0	3	0	3	3
PCV2	21	0	3	0	3	3
PRRSV	14	0	3	0	3	3

*Serum samples were obtained from the source as outlined previously [83].

CSFV E2 and E^{rns} antibodies detection using the ICS and commercial ELISA kits

The correlation between ICS and ELISA kits was then examined. Fifty-seven serum samples were randomly obtained from CSFV-positive farms positive for E2 antibody using ELISA kits (Bionote and IDEXX ELISA kits) and CSFV-negative farms that were tested to be negative for E^{rns} antibody using two commercial ELISA kits (Indical Bioscience and Thermo Fisher Scientific ELISA kits). However, one serum sample was in poor condition during analysis and was positive for E2 antibody but could not be pinpointed as the band for E^{rns} antibody detection. Therefore, the total number of E2 and E^{rns} antibody analysis samples was 57 and 56, respectively. The results in Table 8 indicated that E2 positive proportions of the ICS corresponding to the Bionote and IDEXX ELISA kits were 100% (34 of 34) and 94.1% (32 of 34), respectively. The sensitivity and specificity of ICS corresponding to the Bionote ELISA kits were 89.5% (34 of 38) and 100% (19 of 19), respectively. In addition, the high sensitivity and specificity of ICS corresponding to the IDEXX ELISA kits were 100% (32 of 32) and 92.0% (23 of 25), respectively. A high agreement rate of ICS was determined to be 93.0% (53 of 57) and 96.5% (55 of 57), corresponding to the Bionote and IDEXX ELISA kits, respectively. In Table 9, the E^{rns} positive percentages of ICS corresponding to the Indical Bioscience and Thermo Fisher Scientific ELISA kits were 86.2% (25 of 29) and 79.3% (23 of 29), respectively. ICS sensitivity for E^{rns} antibody detection corresponding to the ELISA kits was 100% for the Indical Bioscience ELISA kits (25 of 25) and the Thermo Fisher Scientific ELISA kits (23 of 23). ICS showed high specificity for E^{rns} detection at 87.1%, corresponding to the Indical Bioscience ELISA kits (27 of 31) and 81.8%, conforming to the Thermo Fisher Scientific ELISA kits (27 of 33). The agreement rates of ICS with the Indical Bioscience and Thermo Fisher Scientific ELISA kits were 92.9% (52 of 56) and 89.3% (50 of 56), respectively.

ICS also strongly agreed with commercial ELISA kits in detecting E2 and E^{rns} antibody responses to the subunit CSFV vaccine. This was observed in serum samples collected from pigs immunized twice with the CSFV E2 subunit vaccine at 40 and 60 days of age. As presented in Table 10, all swine sera tested negative for the E^{rns} antibody using commercial Bionote and IDEXX ELISA kits and ICS. The E2 antibody detection results using ICS aligned with those obtained from the commercial Bionote and IDEXX ELISA kits using serum samples collected at 40 dpv. This confirmation suggested that the

ICS could detect the E2 antibody at a late stage in subunit-vaccinated pigs (Table 10). Taken together, ICS was coincident at a high rate with the commercial ELISA kits used for detecting E2 and E^{rns} antibodies, which would emerge as a superior choice.

Table 8. Correlation between the ICS and the two commercial ELISA kits for detecting CSFV E2 antibody

ICS		C1*		C2*		Total
		Positive	**Negative**	**Positive**	**Negative**	
E2	Positive	34	0	32	2	34
	Negative	4	19	0	23	23
	Total	38	19	32	25	57

*C1, commercial Bionote ELISA kit; C2, commercial IDEXX ELISA kit.

Table 9. Correlation between the ICS and the two commercial ELISA kits for detecting CSFV E^{rns} antibody

ICS		C3*		C4*		Total
		Positive	**Negative**	**Positive**	**Negative**	
E^{rns}	Positive	25	4	23	6	29
	Negative	0	27	0	27	27
	Total	25	31	23	33	56

*C3, commercial Indical Bioscience ELISA kit; C4, commercial Thermo Fisher Scientific ELISA kit.

Table 10. Detection of E2 and E^{rns} antibodies using commercial ELISA kits and the ICS in vaccinated pigs

Pig ID	Antibody detection																	
	E2									E^{rns}								
	0 dpv*			20 dpv**			40 dpv			0 dpv*			20 dpv**			40 dpv		
	C1	C2	ICS	C1	C2	ICS	C1	C2	ICS	C3	C4	ICS	C3	C4	ICS	C3	C4	ICS
#1	–	–	–	+	–	–	+	+	+	–	–	–	–	–	–	–	–	–
#2	–	–	–	+	–	–	+	+	+	–	–	–	–	–	–	–	–	–
#3	–	–	–	+	–	–	+	+	+	–	–	–	–	–	–	–	–	–
#4	–	–	–	+	–	+	+	+	+	–	–	–	–	–	–	–	–	–
#5	–	–	–	+	–	+	+	+	+	–	–	–	–	–	–	–	–	–
#6	–	–	–	+	–	–	+	+	+	–	–	–	–	–	–	–	–	–

*First vaccination shoot; **booster shot; C1, commercial Bionote ELISA kit; C2, commercial IDEXX ELISA kit; C3, commercial Indical Bioscience ELISA kit; C4, commercial Thermo Fisher Scientific ELISA kit; –, negative; +, positive.

Diagnostic capability of the ICS in differentiating infected from vaccinated animals using sera of pigs vaccinated with the chimeric vaccine candidates vGPE$^-$/PAPeV E^{rns} and vGPE$^-$/PhoPeV E^{rns}

The swine serum samples obtained from two independent experiments from Chapter I, were used to assess the applicability of ICS for DIVA. In detail, serum samples from 26 pigs in the optimal infectious dose experiments were tested at 0 and 21 dpv using the ICS (Table 11). As shown in Table 11, all serum samples were negative for E2 and E^{rns} antibodies at 0 dpv, consistent with SNT results. In addition, CSFV E2 neutralizing antibody was detected in the serum samples at 21 dpv of pigs immunized with vGPE$^-$/PAPeV E^{rns} (6 of 9) or vGPE$^-$/PhoPeV E^{rns} (4 of 4). At the same time, there were undetectable against E^{rns} antibody in all pigs vaccinated with each chimeric virus. Conversely, the E^{rns} antibody was detected in almost all serum samples from pigs vaccinated with conventional vGPE$^-$ strain, whereas only 3 of 9 swine serum samples showed positive results for the E2 antibody. Likewise, serum samples taken at different stages postvaccination from pigs vaccinated with commercial LAV GPE$^-$ were used to evaluate the performance of ICS. Most serum samples exhibiting high SNT titers showed a high positive rate for either E2 or E^{rns} antibody or both, whereas samples with low SNT titers or a vaccination period <45 days were negative using ICS. Here, the anti-CSFV E2 antibody was detected in 18 of 24 serum samples, whereas 14 of 24 tested sera were positive for E^{rns} antibody, primarily observed between 90 dpv and before slaughtering (Table 12). Furthermore, antibody responses to E2 and E^{rns} were assessed using serum samples collected at 0 and 7 dpv, and 14 dpc (or 21 dpv) in challenge studies, in which each chimeric virus candidate (vGPE$^-$/PAPeV E^{rns} or vGPE$^-$/PhoPeV E^{rns}) or a conventional LAV vGPE$^-$ was independently administered 7 days before challenge with the moderately virulent CSFV vALD-A76 strain and continuously monitored for 14 dpc. As presented in Table 13, the results showed that the E2 and E^{rns} antibodies were undetected in all serum samples at 0 and 7 dpv. However, an increasing tendency of the E2 antibody was confirmed with positive results in several serum samples from pigs vaccinated with each chimeric virus at 14 dpc, whereas the E^{rns} antibody was negative for all serum samples, as expected. It is crucial to notice that the E^{rns} antibody was detected only in all pigs immunized with conventional LAV and the control groups (unvaccinated pigs), whereas it was negative for those vaccinated with each chimeric virus mentioned

above at 14 dpc. The results of E2 antibody detection in sera from unvaccinated pigs were negative in all serum samples at 14 dpc. In contrast, the E2 antibody against CSFV vGPE^{-} required a longer time to reach the detection threshold on the ICS than the SNT test.

Table 11. Detection of E2 and E^{rns} antibody responses from serum samples in pigs inoculated with vGPE^{-}, vGPE^{-}/PAPeV E^{rns}, or vGPE^{-}/PhoPeV E^{rns} at different doses of $TCID_{50}$

Virus	Inoculation dose ($TCID_{50}$)	Pig ID*	0 dpv			21 dpv		
			E2**	E^{rns}**	SNT	E2**	E^{rns}**	SNT
vGPE^{-}/PAPeV E^{rns}	$10^{3.0}$	#312	–	–	<1	–	–	–
		#313	–	–	<1	+	–	1
		#314	–	–	<1	–	–	–
	$10^{4.0}$	#315	–	–	<1	+	–	8
		#316	–	–	<1	–	–	1
		#317	–	–	<1	+	–	2
	$10^{5.0}$	#318	–	–	<1	+	–	8
		#319	–	–	<1	+	–	32
		#320	–	–	<1	+	–	4
vGPE^{-}/PhoPeV E^{rns}	$10^{3.0}$	#354	–	–	<1	+	–	2
		#355	–	–	<1	–	–	<1
		#356	–	–	<1	+	–	2
	$10^{4.0}$	#357	–	–	<1	–	–	1
		#358	–	–	<1	–	–	1
		#359	–	–	<1	–	–	1

Table 11 (*cont*)**.** Detection of E2 and E^{rns} antibody responses from serum samples in pigs inoculated with $vGPE^{-}$, $vGPE^{-}$/PAPeV E^{rns}, or $vGPE^{-}$/PhoPeV E^{rns} at different doses of $TCID_{50}$

Virus	Inoculation dose ($TCID_{50}$)	Pig ID*	0 dpv			21 dpv		
			E2**	E^{rns}**	SNT	E2**	E^{rns}**	SNT
$vGPE^{-}$/PhoPeV E^{rns}	$10^{5.0}$	#360	−	−	<1	+	−	8
		#362	−	−	<1	+	−	32
$vGPE^{-}$	$10^{3.0}$	#303	−	−	<1	–	+	1
		#304	−	−	<1	–	–	<1
		#305	−	−	<1	–	+	4
	$10^{4.0}$	#306	−	−	<1	–	+	4
		#307	−	−	<1	–	+	4
		#308	−	−	<1	+	+	8
	$10^{5.0}$	#309	−	−	<1	+	+	4
		#310	−	−	<1	–	–	1
		#311	−	−	<1	+	+	8

*Serum samples were obtained from the source as outlined in Chapter I; **antibody detection using ICS; −, negative; +, positive.

Table 12. Detection of E2 and E^{rns} antibody responses from serum samples in pigs post inoculated with the commercial vaccine CSFV GPE^{-} strain at different time points using the ICS and SNT

Pig ID	Serum at dpv	Antibody detection using ICS		SNT
		E2	E^{rns}	
#135	0	–	–	2*
	45	+	+	128
	90	+	+	64
	Before slaughtering	+	+	128
#139	0	–	–	2*
	45	+	+	128
	90	+	+	256
	Before slaughtering	+	+	512
#168	0	+	–	32*
	45	–	+	16
	90	+	+	16
	Before slaughtering	+	+	16
#179	0	+	+	32*
	45	–	+	4
	90	+	+	4
	Before slaughtering	+	+	16
#182	0	+	–	32*
	45	–	–	8
	90	+	–	16
	Before slaughtering	+	–	16
#190	0	+	–	64*
	45	–	–	16
	90	+	+	32
	Before slaughtering	+	–	16

*Maternally derived antibodies; –, negative; +, positive.

Table 13. Detection of E2 and E^{rns} antibody responses from serum samples in pigs vaccinated with vGPE^{-}, vGPE^{-}/PAPeV E^{rns}, or vGPE^{-}/PhoPeV E^{rns} in the challenge studies using the ICS and SNT

Virus	Pig ID*	0 dpv			7 dpv			14 dpc		
		E2**	E^{rns}**	SNT	E2**	E^{rns}**	SNT	E2**	E^{rns}**	SNT
vGPE^{-}/PAPeV E^{rns}	#353	–	–	<1	–	–	<1	–	–	2
	#352	–	–	<1	–	–	<1	+	–	16
	#351	–	–	<1	–	–	<1	–	–	4
vGPE^{-}/PhoPeV E^{rns}	#373	–	–	<1	–	–	<1	+	–	4
	#372	–	–	<1	–	–	<1	+	–	8
	#371	–	–	<1	–	–	<1	+	–	4
vGPE^{-}	#350	–	–	<1	–	–	<1	–	+	4
	#349	–	–	<1	–	–	<1	+	+	16
	#348	–	–	<1	–	–	<1	–	+	8
Nonvaccination	#347	–	–	<1	–	–	<1	–	+	<1
	#346	–	–	<1	–	–	<1	–	+	<1
	#345	–	–	<1	–	–	<1	–	+	<1
	#370	–	–	<1	–	–	<1	–	+	<1
	#369	–	–	<1	–	–	<1	–	+	<1
	#368	–	–	<1	–	–	<1	–	+	<1

*Serum samples were obtained from the source as outlined in Chapter I. Briefly, pigs were immunized at a dose of $10^{4.0}$ $TCID_{50}$ with each chimeric vaccine candidate, $vGPE^{-}$, and unvaccinated pigs. All pigs were challenged with the moderately virulent CSFV vALV-A76 strain at a dose of $10^{6.0}$ $TCID_{50}$ and monitored for 14 days; **antibody detection using ICS; −, negative; +, positive.

Discussion

Vaccination programs employing conventional CSFV LAVs have been demonstrated to protect pigs against CSFV infection and clinical signs in early-stage vaccination [50,65]. Nevertheless, these conventional vaccines cannot resolve the challenges associated with the DIVA concept and the interference of MDA. Various CSFV marker vaccines have been developed to address this challenge, such as subunit vaccines or chimeric pestiviruses designed to fulfill the DIVA strategy for controlling and eradicating CSF [17,18,70,71]. With the improvement of vaccine candidates, DIVA diagnostic tools using ELISA kits for detecting CSFV E^{rns} antibody or monitoring the induction of neutralizing antibodies using some commercial ELISA kits for E2 antibody detection have been developed and employed as adjunctive diagnostic assessments together with the E2 subunit vaccine and chimeric pestiviruses [23,69]. However, ELISA procedures are time-consuming and require skilled personnel, which might not be suitable for rapid field testing if E2 and E^{rns} antibody detection needs to be evaluated simultaneously with a large sample number in the field. Colloidal gold ICS have been established and are a valuable diagnostic tool for monitoring antibodies against CSFV in vaccinated pigs [72–74]. Compared to alternative antibody detection methodologies such as SNT or ELISA, ICS is notably user-friendly, rapid, and easily transportable [23,72]. Thus far, ICS development has preferably focused on detecting antibodies against the CSFV E2 protein, a powerful tool for the CSFV antibody assessment [73,74]. Meanwhile, ICS development for CSFV E^{rns} antibody detection for the DIVA diagnostic approach still has no specific innovation [23]. Furthermore, considering the benefits of transient protein expression in plant-derived systems [81,87,88], a novel ICS has been developed for the simultaneous detection of dual CSFV E2 and E^{rns} antibodies. This study established ICS based on colloidal gold recombinant E2 and E^{rns} proteins derived from *N. benthamiana* plants to rapidly evaluate CSFV antibodies and integrate DIVA diagnostic features.

In protein manipulation, previous studies have indicated that plant-derived transient expression enhances high protein expression and yield, immunogenicity in animal experiments, and high binding affinity for antibody detection absent in certain conventional expression systems [74,87,89]. Compared to the transgenic approach for CSFV E2 protein expression using baculovirus [59,73], mammalian cells [90,91], or the *E. coli* system [72,92], the CSFV E2-expressing transgenic plant-derived system was

preferable in terms of a large amount and stable expression of the target protein and inexpensiveness, as in recent studies [74,81,84,88,89]. This approach facilitated the purification of recombinant E2 from *N. benthamiana* extracts. Although the E2 protein is typically dimeric form, earlier investigations [81] have confirmed a multimeric form of E2 protein expressed in plant tissue through glycosylation. A few systems have been established for recombinant E^{rns} protein expression in *E. coli* [92,93], baculovirus [59], or yeast [94,95], but there are several concerns regarding inappropriate posttranslational modifications, particularly the absence or low proportion of glycosylation in E^{rns} or E2 protein that may influence the binding affinity of antibodies [96]. The most important feature of CSFV E2 and E^{rns} is their heavy glycosylation, particularly E^{rns}, which contains seven putative *N*-linked glycosylation sites. *N*-glycosylation results in a complex mixture of neutral and monosialylated multiantennary *N*-glycans, accounting for nearly half of the molecular mass of mature glycoproteins [96,97]. Interestingly, in terms of *N*-glycosylation, a previous study [80] reported that *N. benthamiana* plant-based expression platforms exhibit remarkable versatility in that they can effectively accommodate transient and stable engineering of the *N*-glycan processing pathways without inducing cell death or generating adverse growth phenotypes associated with biomass production, protein expression or yield, features lacking in conventional expression systems. In this study, protein expression using a plant-derived system was employed as a first report for E^{rns} protein expression in which the yielded E^{rns} protein had a molecular mass of ~45 kDa, comparable to the mature E^{rns} protein in previous studies [98,99]. Although it was speculated that E2 and E^{rns} proteins would be highly glycosylated in the plant expression system, the purified proteins were successfully conjugated with colloidal gold in this study to develop the ICS, thereby enhancing their availability.

This study collected various sera to assess the sensitivity and specificity of ICS and its correlation with commercial ELISA kits. As mentioned, ICS showed high sensitivity or specificity for detecting CSFV E2 and E^{rns} antibodies under different vaccination or infection backgrounds, particularly E^{rns} detection, even at low SNT titers (Tables 6 and 11−13), which would be difficult to confirm through ELISA kits. The performance of ICS exhibited high coincidence rates with ELISA kits detecting E2 and E^{rns} antibodies. However, the preference for detecting E2 antibodies was evident, particularly later when the E2 subunit vaccine was employed in this study (Table 10).

Despite ICS exhibiting sensitivity with SNT titers of 16 with serum samples from pigs vaccinated with the modified live LOM strain (Table 6), it surprisingly demonstrated high sensitivity in detecting E2 antibodies in serum samples from pigs vaccinated with commercial conventional CSFV LAV GPE$^-$ or chimeric vaccines, even at lower SNT titers of 16 (Tables 11−13). Although this study confirmed high sensitivity and specificity across a diverse range of sera, the sample size was small. Therefore, future studies should employ larger sample sizes to assess the reproducibility of ICS. Given the impact of storage temperature, time, and manufacturing batches on the reliability of detection, further research is essential to evaluate the repeatability and stability of ICS. In addition, because the dependence of CSFV-specific antibody responses on several factors, such as CSFV infection with different genotypes, the induction of E2- or E^{rns}-specific antibody [100], and the failure of vaccination or the delay of antibody responses against CSFV induced by PRRSV [101,102], a comprehensive approach involving a combination of various methods and continuous monitoring is essential to accurately determine the antibody response against CSFV.

ICS was used to evaluate the DIVA profiles of two chimeric pestiviruses, generated based on an infectious cDNA clone derived from the LAV GPE$^-$ strain. These chimeric viruses possess the E^{rns} glycoprotein, which is phylogenetically distant from CSFV, and are potential DIVA vaccine candidates. Serum samples obtained from optimal vaccination dose experiments and vaccine-efficient studies were reused to evaluate the DIVA profiles of these chimeric vaccine candidates. In the optimal vaccination dose experiment, successful detection of anti-CSFV E^{rns} antibodies in serum samples from conventionally vaccinated pigs was confirmed, whereas all chimeric-vaccinated pigs were negative, indicating the effectiveness of this ICS in detecting specific CSFV E^{rns} antibodies. Meanwhile, the presence of an E2-specific antibody indicated that antibody responses depended on the individual and the time point postvaccination (Table 12). Significantly, the DIVA capability of chimeric vaccine candidates was validated in vaccine efficacy studies focusing on CSFV E^{rns} detection, by which ICS successfully distinguished chimeric-vaccinated animals from infected and conventionally vaccinated animals. The outcomes obtained provide valuable insights into the potential of this ICS to address the limitations of previous ICS that lack DIVA diagnostic capabilities with E^{rns} antibody detection [72–74]. Although CSFV E^{rns} is used as a negative marker to evaluate

the feasibility of ICS for DIVA in this study, future studies should concentrate on developing a serological antibody assay capable of detecting antibodies against recombinant PAPeV E^{rns} and PhoPeV E^{rns}. This development will facilitate the implementation of marker vaccine candidates vGPE^{-}/PAPeV E^{rns} and vGPE^{-}/PhoPeV E^{rns} to control and eradicate CSF in the future. In addition, DIVA application with E^{rns} antibody detection was not feasible to evaluate CSF-free status individually in animals, a limitation of the sera used in this study. Instead, detection of E^{rns} CSFV-specific antibodies was suggested for implementation at the herd level, as validated in previous studies [66,103]. Therefore, further investigations are necessary to comprehensively validate ICS specificity and sensitivity across larger populations under various field conditions. Because serum samples derived from CSFV genotype 1 were mainly used in this study, evaluating its performance against diverse strains, genotypes, and its potential cross-reactivity with antibodies from other swine pathogens in further studies will enhance its reliability and applicability in practical DIVA strategies.

Until now, the gold standard for determining antibodies against CSFV is SNT. However, existing commercial ELISA kits evaluate E2 or E^{rns} separately, which incurs additional costs and labor and is more time-consuming. Although ICS development for antibody detection has been established, previous efforts have primarily focused on detecting CSFV E2 protein antibodies. This study is a pioneering effort to develop an ICS capable of detecting E2 and E^{rns} antibodies simultaneously. This ICS addresses the prior limitation of being unable to differentiate infected from vaccinated animals from previous studies [73,74]. Extensive studies and long-term monitoring are also essential to evaluate the DIVA potential of subunit or chimeric vaccines that contribute significantly to CSF surveillance and control.

Brief summary

It is essential to consider a practical antibody test to successfully implement marker vaccines and validate vaccination efficacy against CSFV. The test should include a serological antibody assay, combined with a tool for DIVA capability. The ICS has been exclusively designed for detecting CSFV E2 antibodies while lacking in detecting E^{rns} antibodies, which can be employed and satisfy DIVA strategy. This study developed a novel ICS for detecting CSFV E2/E^{rns} dual-antibody. The effectiveness of ICS in evaluating the DIVA capability of two novel chimeric pestivirus vaccine candidates was assessed. Recombinant E2 or E^{rns} protein was transiently expressed in the plant *N. benthamiana* using *A. tumefaciens*. ICS was subsequently assembled, and goat anti-rabbit IgG and recombinant CSFV E2 or E^{rns} protein were plated onto the nitrocellulose membrane as control and test lines, respectively. The sensitivity and specificity of ICS were evaluated using sera with different neutralizing antibody titers or positive for antibodies against CSFV and other pestiviruses. The coincidence rates for detecting E2 and E^{rns} antibodies between ICS and commercial ELISA kits were also computed. ICS performance for DIVA capability was evaluated using sera from pigs vaccinated with conventional vaccine or chimeric vaccine candidates. As a result, two recombinant proteins were successfully expressed, and each was conjugated with gold nanoparticles. ICS demonstrated high sensitivity in identifying CSFV E2 and E^{rns} antibodies, even at the low neutralizing antibody titers. No cross-reactivity with antibodies from other pestiviruses was confirmed using ICS. There were high agreement rates of 93.0% and 96.5% between ICS and two commercial ELISA kits for E2 antibody testing. ICS also achieved strong coincidence rates of 92.9% and 89.3% with two commercial ELISA kits for E^{rns} antibody detection. ICS confirmed the absence of CSFV E^{rns}-specific antibodies in sera from pigs vaccinated with chimeric vaccine candidates. E2 and E^{rns} proteins derived from the plant showed great potential and can be used to engineer a CSFV E2/E^{rns} dual-antibody ICS. The ICS was also highly sensitive and specific for detecting CSFV E2 and E^{rns} antibodies. Significantly, ICS can fulfill the DIVA concept by incorporating chimeric vaccine candidates.

Chapter III

Assessment of the safety profile of chimeric marker vaccine against classical swine fever: reversion to virulence study

Introduction

Vaccination is a crucial strategy for controlling CSF outbreaks, with LAVs commonly employed due to their efficacy [2,24]. However, LAVs carry inherent risks, including the potential for reversion to virulence, where the vaccine strain regains its ability to cause the re-emergence of CSF after introducing the vaccine in the field [13,14]. A previous study has cautioned about the reversion to virulence of an LAV, FlagT4v, whose virulence was restored during five successive passages in piglets [26]. This risk is further heightened by a recent study, which demonstrated that a chimeric CSFV vSM/CE2 containing the E2 gene of the vaccine C-strain on the genetic background of the virulent CSFV strain Shimen, was reversed to virulence after serial passages in PK15 cells, and the increased virulence of its mutants was also confirmed in the experimental infection in pigs [27]. Despite the extensive utilization of the LAV GPE$^-$ strain in the Asian and Pacific regions for numerous years [20,31], the confirmed risk of reversion to virulence of LAV GPE$^-$ strain after 11 serial passages in pigs has been affirmed in a previous study [37], underscoring the importance of assessing safety profile, particular in vaccine reversion to virulence [20].

The structural glycoprotein E^{rns}, unique to pestiviruses and an essential component of the viral envelope, has multifaceted functions critical for viral replication, pathogenesis, and immune evasion [2,6]. E^{rns} possesses RNase activities, enabling it to degrade viral and host RNAs and thereby suppressing the host's innate immune response and facilitating viral persistence within the host. Despite its crucial functions in viral biology, E^{rns} also plays a significant role as an antigen in diagnosing and developing genetically engineered vaccines. Considering that the ability of E^{rns} to elicit neutralizing antibodies is not the primary component [7,9] and is a suitable candidate for epitope substitution [57], several chimeric pestiviruses possessing the E^{rns} which is genetically related within *Pestivirus* genus, have been intensively developed to improve CSF marker vaccine candidates with the potential to combine the efficacy of LAVs and DIVA properties [18,19]. Nevertheless, additional assessments of these candidates, covering aspects such as safety profiles, particularly in the potential for reversion to virulence, have not yet been conducted [18,19]. Notably, a recent study has confirmed the reversion to virulence associated with the CSFV E^{rns} glycoprotein [104], in which the recombinant virus C-W300G derived from the virulent CSFV Alfort/Tübingen strain, possessing a mutant that

eliminated the RNase activity of E^{rns}, initially demonstrated attenuation in pigs; however, subsequent inoculation of the reisolated virus in pigs revealed a rapid reversion to virulence and restored the wild-type characteristics. In this scenario, the insufficiency of safety assessments raises concerns about the possibility of regaining virulence, especially concerning chimeric pestivirus vaccines, designed to express foreign genes from related pestiviruses [20].

Two novel chimeric marker vaccine candidates, vGPE^{-}/PAPeV E^{rns} and vGPE^{-}/PhoPeV E^{rns}, were developed and their biological properties and DIVA capabilities have been confirmed, as detailed in Chapters I and II, respectively. These two chimeric viruses were generated based on the backbone of the LAV vGPE^{-} strain [37,40], and possessed the glycoprotein E^{rns} from either PAPeV [28] or PhoPeV [58], both of which are distantly related to CSFV. Although serial passages in SK-L cells confirmed high viral growth and genetic stability, their safety characteristics have not been validated through serial passages in pigs. This study continued a series of research projects aimed at evaluating the safety profiles of the two chimeric marker vaccine candidates, particularly in the potential reversion to virulence.

Materials and Methods

Cells and viruses

The SK-L cells [41] was cultured in EMEM (Nissui Pharmaceutical) supplemented with 0.295% TPB (Becton Dickinson), 10 mM BES (Sigma-Aldrich), sodium bicarbonate (Nacalai Tesque), and 10% HS (Thermo Fisher Scientific). SK-L cells were employed to evaluate viral growth kinetics, virus titration, and serological testing. Cultured cells were incubated at 37 °C with 5% CO_2. The SK6-MxLuc cell used in the IFN bioassay, harboring an Mx/Luc reporter gene, was cultured in EMEM supplemented with 10 mM BES and 7% HS [105,106].

An engineered clone of CSFV-modified LAV (vGPE$^-$) derived from pGPE$^-$ [37,40] was used. Two chimeric marker vaccine candidates vGPE$^-$/PAPeV E^{rns} and vGPE$^-$/PhoPeV E^{rns}, possessing PAPeV E^{rns} and PhoPeV E^{rns}, respectively, were generated and evaluated that were mentioned in Chapters I and II.

Virus titration

To determine viral titers, blood samples or 10% tonsil homogenates were added to SK-L cells cultured in 6-well plates. Serially diluted 10-fold samples (either blood samples or 10% tonsil homogenates) were cultured simultaneously in 96-well plates and incubated for 96 h at 37 °C in 5% CO_2. The detailed procedure was previously described [45] and explained in Chapter I.

Viral growth kinetics

The growth kinetics of the vGPE$^-$ virus and the two chimeric marker vaccine candidates in SK-L cells were assessed by introducing them to confluent cell monolayers at a MOI of 0.001. After inoculation, SK-L cells were cultured at 37 °C with 5% CO_2. Cell supernatants were collected at 0, 1, 2, 3, 4, 5, 6, and 7 dpi. Viral titers were determined in triplicate using SK-L cells, according to the abovementioned virus titration.

Bioassay for IFN-α/β measurement

The bioassay was conducted following previously established methods for measuring swine IFN-α/β [105,106]. In summary, supernatants from SK-L cells infected with each virus were rendered inactive using an ultraviolet cross-linker (DNA-FIX DF-

254; ATTO, Tokyo, Japan) before being introduced to SK6-MxLuc cells. The recombinant swine IFN-α/β produced in a previous study was employed as the standard [42]. Cell lysates were generated using 100 μL passive lysis buffer, and firefly luciferase activities were quantified using the Dual-Luciferase Reporter Assay System and POWERSCAN®4 (Agilent Technologies International Japan Ltd.). Results were documented across three independent trials, each executed in duplicate.

Sequencing

The entire genome of the recovered vGPE$^-$ virus or each chimeric marker vaccine candidate in the tonsil homogenate samples from inoculated pigs was verified as previously described [37] and detailed extensively in Chapter I.

Animal use

Pigs were purchased from a CSFV-free farm (Yamanaka Chikusan) in Hokkaido and confirmed to be free of antibodies against CSFV, as outlined in Chapter I.

Reversion to virulence study through experimental infection in pigs

The safety test involved serial passage of viruses in pigs, as illustrated in Figure 1. Briefly, either each chimeric marker vaccine candidate (vGPE$^-$/PAPeV E^{rns} or vGPE$^-$/PhoPeV E^{rns}) or vGPE$^-$ was introduced via intramuscular injection of 2 mL cell culture supernatant of each virus into two 2-week-old crossbred Landrace × Duroc × Yorkshire SPF pigs (Yamanaka Chikusan) per passage at a dose of $10^{4.0}$ $TCID_{50}$, recommended as equivalent to one dose of the vaccine in Chapter I. Twenty-two pigs were employed in this study. Among them, 4 pigs were injected with vGPE$^-$/PAPeV E^{rns} within two passages, 8 pigs were injected with vGPE$^-$/PhoPeV E^{rns} within four passages, and 10 pigs were injected with vGPE$^-$ within five passages. All pigs were monitored daily for clinical signs according to a scoring system for 7 days [48]. Blood samples were collected in tubes containing EDTA (Venoject II VP-NA050K, Terumo) at 0, 2, 3, 4, 5, 6, and 7 dpi. Serum samples were collected using tubes containing a blood coagulation factor (Venoject II VP-P075K, Terumo) at 0 and 7 dpi. The levels of CSFV-specific neutralizing antibodies of pigs at 0 and 7 dpi were evaluated by a serological test. All survival pigs were euthanized at 7 dpi. Tonsil samples were collected aseptically. For virus titration, 10% organ homogenates were prepared and stored at –80 °C. Virus titers

were measured and displayed as $TCID_{50}$/mL (blood) or $TCID_{50}$/g (tissue) [47]. If no virus recovery was found after each passage, the experiment was ended. If virus recovery was confirmed, pooled tonsil homogenates were used to inoculate the next two pigs of the same age and origin by the same route as before. The serial passage in pigs was carried out five times in total from the start of the first vaccination experiment.

Serum neutralization test

The assay was conducted by employing the luciferase-based method, which was previously outlined [49] and described thoroughly in Chapter I.

Statistical analysis

Statistical analyses of the data were performed using one-way analysis of variance, followed by Student's *t*-test with Bonferroni correction.

Ethics statement

Animal experiments were approved by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine, Hokkaido University (approval no. 23-0029, approved on March 23, 2023), and performed according to the guidelines of this committee. Animals reaching the humane endpoint were euthanized through intracardial injection of thiopental sodium (Ravonal®; Nipro ES Pharma Co., Ltd.) after deep sedation with isoflurane (Fujifilm Wako Pure Chemical Co., Ltd.), as described previously. The experiments took place in animal facilities accredited by the AAALAC International.

Results

Attenuation of chimeric viruses during serial passages in pigs

To determine whether the two chimeric marker vaccine candidates, vGPE$^-$/PAPeV E^{rns} or vGPE$^-$/PhoPeV E^{rns}, and the vGPE$^-$ strain can regain pathogenicity in pigs through forced transmission from pig to pig, the virus underwent serial passages in pigs via injection of virus recovered from infected animals. As a result, passaging of each virus or tonsil homogenates from vaccinated pigs was performed 2, 4, and 5 times for vGPE$^-$/PAPeV E^{rns}, vGPE$^-$/PhoPeV E^{rns}, and vGPE$^-$, respectively (Figure 11). Both chimeric marker vaccine candidates vGPE$^-$/PAPeV E^{rns} and vGPE$^-$/PhoPeV E^{rns} complied with the test due to the absence of virus recovery after the second and fourth passages, respectively. However, the vGPE$^-$ virus was still recovered until the fifth passage in pigs.

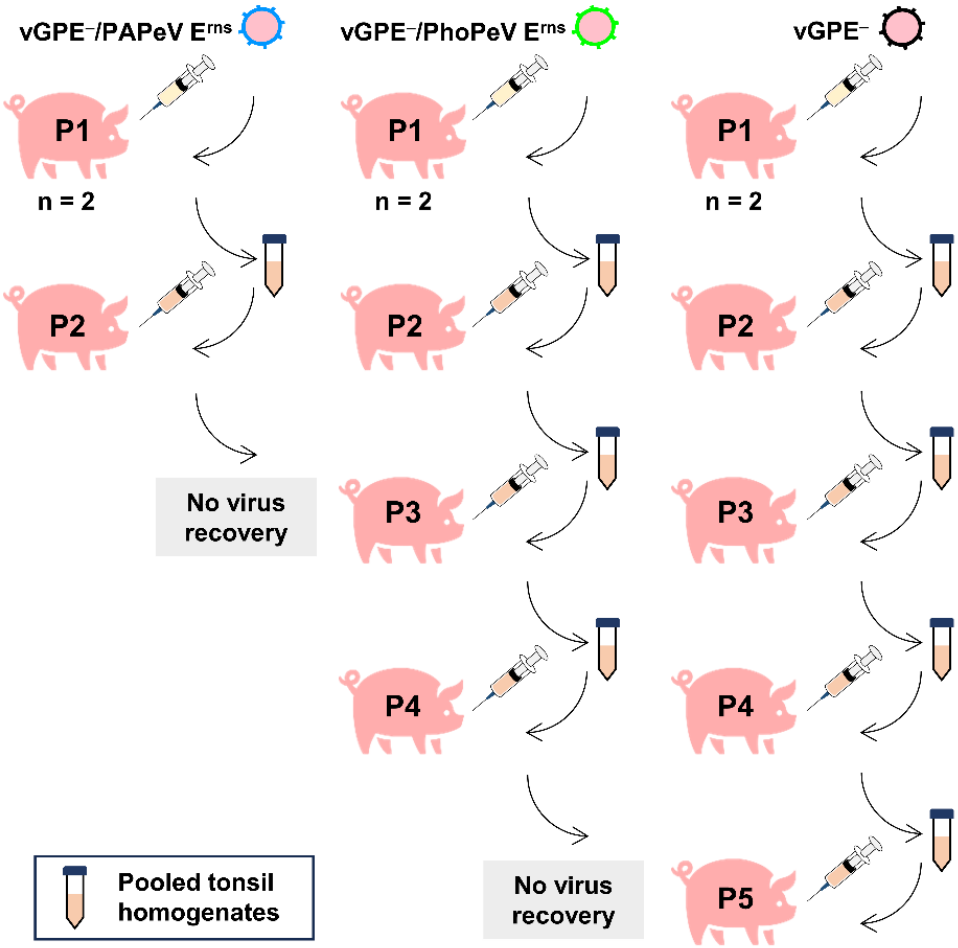


Figure 11. Study design for serial passages of viruses in pigs. Virus seeds, including vGPE⁻/PAPeV E^{rns}, vGPE⁻/PhoPeV E^{rns}, and vGPE⁻, were independently inoculated into two pigs per passage. A total of four pigs received injections of vGPE⁻/PAPeV E^{rns} over 2 passages (left side). Other eight pigs were injected with vGPE⁻/PhoPeV E^{rns} over 4 passages (center). Ten pigs were injected with vGPE⁻ over 5 passages (right side).

In each passage in pigs, the clinical signs of inoculated pigs were monitored daily until 7 dpi, and virus recovery in blood samples was determined daily between days 2 and 7 during the experiment, as shown in Table 14. Based on a clinical scoring system [48], there were no abnormalities in clinical signs in all pigs of each passage. The results of the SNT titer also confirmed the absence of neutralizing antibodies against CSFV in the naïve pigs at 0 and 7 dpi. The absence of viral replication in blood samples (Table 14) in each passage in pigs inoculated with either vGPE^{-}/PAPeV E^{rns} or vGPE^{-}/PhoPeV E^{rns} was evidenced by virus titration. Transient viremia was confirmed in the second passage in one pig inoculated with the recovered P1/vGPE^{-} virus at 6 dpi. Although viremia was undetectable in pigs inoculated with the recovered vGPE^{-} virus from the second to the fourth passage, the presence of the virus was reconfirmed in the fifth passage. In detail, the virus recovery was confirmed positive in 6-well plates but lower than the detection limit of $10^{0.8}$ $TCID_{50}$/mL in 96-well plates in blood samples at 5, 6, and 7 dpi in two pigs inoculated with the recovered P4/vGPE^{-} virus. At 7 dpi in each passage, pigs were euthanized, and virus recovery was then determined in tonsil homogenates. After the first passage, virus titers in the tonsils of inoculated pigs were $<10^{3.0}$ $TCID_{50}$ (Table 14) in one pig inoculated with either vGPE^{-}/PAPeV E^{rns} or vGPE^{-}/PhoPeV E^{rns}; meanwhile, virus recovery was confirmed positive in 6-well plates but lower than the detection limit of $10^{1.8}$ $TCID_{50}$/g in 96-well plates in the remaining pig in each group. Virus recovery was undetectable in tonsil homogenates after the second passage in two pigs inoculated with P1/vGPE^{-}/PAPeV E^{rns} and one pig inoculated with P1/vGPE^{-}/PhoPeV E^{rns}. Although the remaining pig inoculated with P1/vGPE^{-}/PhoPeV E^{rns}, was confirmed positive in 6-well plates for virus recovery, the virus titer was lower than the detection limit of $10^{1.8}$ $TCID_{50}$/g in 96-well plates. This occurrence was also confirmed in the third passage of pigs inoculated with the recovered P2/vGPE^{-}/PhoPeV E^{rns} virus. However, no virus recovery was confirmed at the fourth passage in pigs inoculated with the P3/vGPE^{-}/PhoPeV E^{rns} virus. In contrast, virus recovery in pigs inoculated with recovered vGPE^{-} virus remained stable ($10^{3.1}$–$10^{5.0}$ $TCID_{50}$) from the first to the fifth passage. These results indicated that two chimeric marker vaccine candidates vGPE^{-}/PAPeV E^{rns} and vGPE^{-}/PhoPeV E^{rns} were attenuated in serial passages in pigs; meanwhile, the vGPE^{-} showed replication stability in pigs without clinical signs.

Table 14. Virus recovery from blood and tissue samples in pigs inoculated with vGPE^{-}/PAPeV E^{rns}, vGPE^{-}/PhoPeV E^{rns}, or vGPE^{-}

Passage (P)	Virus	Pig ID	Clinical signs	Virus recovery								SNT titer at dpi	
				Blood (log_{10} $TCID_{50}$/mL) at dpi							Tissue (log_{10} $TCID_{50}$/g)		
				0	2	3	4	5	6	7	Tonsil	0	7
P1	vGPE^{-}/PAPeV E^{rns}	#384	N/O	–	–	–	–	–	–	–	$10^{2.8}$	<1	<1
		#385	N/O	–	–	–	–	–	–	–	+	<1	<1
	vGPE^{-}/PhoPeV E^{rns}	#386	N/O	–	–	–	–	–	–	–	$10^{2.3}$	<1	<1
		#387	N/O	–	–	–	–	–	–	–	+	<1	<1
	vGPE^{-}	#382	N/O	–	–	–	–	–	–	–	$10^{3.1}$	<1	<1
		#383	N/O	–	–	–	–	–	–	–	$10^{4.6}$	<1	<1
P2	vGPE^{-}/PAPeV E^{rns}	#390	N/O	–	–	–	–	–	–	–	–	<1	<1
		#391	N/O	–	–	–	–	–	–	–	–	<1	<1
	vGPE^{-}/PhoPeV E^{rns}	#392	N/O	–	–	–	–	–	–	–	–	<1	<1
		#393	N/O	–	–	–	–	–	–	–	+	<1	<1
	vGPE^{-}	#388	N/O	–	–	–	–	–	+	–	$10^{3.9}$	<1	<1
		#389	N/O	–	–	–	–	–	–	–	$10^{4.0}$	<1	<1

Table 14 (*cont*)**.** Virus recovery from blood and tissue samples in pigs inoculated with vGPE^{-}/PAPeV E^{rns}, vGPE^{-}/PhoPeV E^{rns}, or vGPE^{-}

Passage (P)	Virus	Pig ID	Clinical signs	Virus recovery								SNT titer at dpi	
				Blood (log_{10} $TCID_{50}$/mL) at dpi							Tissue (log_{10} $TCID_{50}$/g)		
				0	2	3	4	5	6	7	Tonsil	0	7
P3	vGPE^{-}/PhoPeV E^{rns}	#396	N/O	–	–	–	–	–	–	–	–	<1	<1
		#397	N/O	–	–	–	–	–	–	–	+	<1	<1
	vGPE^{-}	#394	N/O	–	–	–	–	–	–	–	$10^{4.0}$	<1	<1
		#395	N/O	–	–	–	–	–	–	–	$10^{4.0}$	<1	<1
P4	vGPE^{-}/PhoPeV E^{rns}	#405	N/O	–	–	–	–	–	–	–	–	<1	<1
		#406	N/O	–	–	–	–	–	–	–	–	<1	<1
	vGPE^{-}	#403	N/O	–	–	–	–	–	–	–	$10^{4.8}$	<1	<1
		#404	N/O	–	–	–	–	–	–	–	$10^{3.6}$	<1	<1
P5	vGPE^{-}	#407	N/O	–	–	–	–	–	–	+	$10^{5.0}$	<1	<1
		#408	N/O	–	–	–	–	+	+	–	$10^{4.8}$	<1	<1

N/O, not observed; –, not isolated in a 6-well plate; +: isolated in a 6-well plate and was lower than the detection limit of $TCID_{50}$ ($10^{0.8}$ $TCID_{50}$/mL for blood samples and $10^{1.8}$ $TCID_{50}$/g for tissue samples) in a 96-well plate.

Amino acid substitutions of chimeric viruses passaged in pigs

To ascertain whether serial passages of the two chimeric marker vaccine candidates and vGPE$^-$ in pigs led to the selection of mutant viruses, the entire genome sequences of the recovered viruses after each passage were examined via direct sequencing of DNA fragments amplified via reverse transcription-PCR, originating from viral RNA. The amino acid substitutions were identified in the viruses recovered after each passage, as shown in Figure 12. Two amino acid substitutions in C protein (L264P) and E1 protein (M660T), were found after the first passage in pigs inoculated with vGPE$^-$/PAPeV E^{rns}. The substitutions were confirmed in the NS5B protein of the P3/vGPE$^-$/PhoPeV E^{rns}, where the substitutions were identified as asparagine to aspartic acid and threonine to isoleucine at positions 3289 and 3842, respectively. In NS5B, threonine to isoleucine substitution at position 3842, arose after the second passage and persisted until the third. After five passages, the P5/vGPE$^-$ virus exhibited a similar threonine to isoleucine substitution at position 3842, as observed in the P2- and P3/vGPE$^-$/PhoPeV E^{rns} (Figure 12). The P5/vGPE$^-$ virus demonstrated quasispecies diversity, indicated by the appearances of additional amino acids resided at L1277M, L2621S, and K3418E, corresponding to the NS2, NS4B, and NS5B proteins, respectively. Amino acid sequences of the recovered viruses, P1/vGPE$^-$/PAPeV E^{rns}, P3/vGPE$^-$/PhoPeV E^{rns}, or P5/vGPE$^-$, were compared to those of vGPE$^-$ and GPE$^-$ vaccine strains, and its parental CSFV ALD strain and recombinant vALD-A76, which was generated from an infectious cDNA clone of the virulent strain ALD-A76 in a previous study [42] and has a different sequence compared to the parental ALD strain (Figure 12). According to the sequence alignment of amino acids, T660 (E1) in P1/vGPE$^-$/PAPeV E^{rns} and I3842 (NS5B) in P3/vGPE$^-$/PhoPeV E^{rns} and P5/vGPE$^-$, were identical to the parental CSFV ALD strain and vALD-A76.

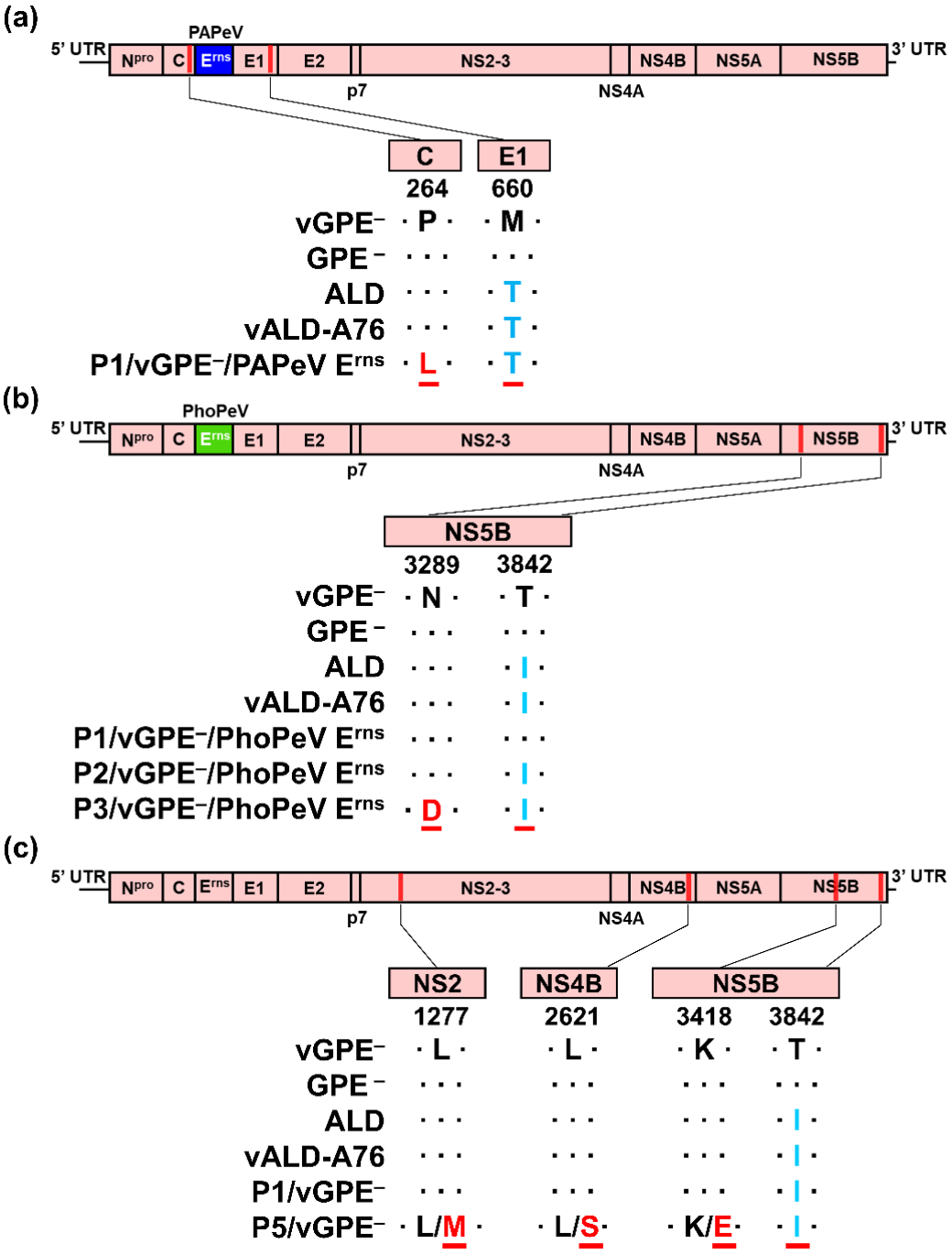


Figure 12. Schematic representation of amino acid substitutions among CSFV strains and passaged viruses in pigs. **(a)** Two amino acid substitutions (P264L and M660T) were found in the P1/vGPE^{-}/PAPeV E^{rns}. **(b)** Two amino acid substitutions (N3289D and T3842I) were found in the P3/vGPE^{-}/PhoPeV E^{rns}. **(c)** The substitution of threonine to isoleucine at position 3842 was identified in P5/vGPE^{-}. The P5/vGPE^{-} virus also exhibited quasispecies diversity with the emergence of amino acid changes at residues L1277M, L2621S, and K3418E. The methionine encoded by the AUG start

codon is defined as position 1, and the subsequent residues are identified according to the backbone vGPE^{-} strain. Two amino acids were found (quasispecies). The periods indicate the identity with the sequence of virus seeds, vGPE^{-}/PAPeV E^{rns}, vGPE^{-}/PhoPeV E^{rns}, or vGPE^{-}. The amino acid sequences different from vGPE^{-} are underlined in red. The amino acid sequences unique to vGPE^{-} and GPE^{-} strains are indicated as periods. The amino acid sequence unique to the parental CSFV ALD and vALD-A76 strains is highlighted in light blue characters.

In vitro growth kinetics of vGPE$^-$ and chimeric viruses

To assess the impact of substituting the glycoprotein E^{rns} on viral growth kinetics and IFN-α/β production in swine cells, multistep growth curves of the two chimeric marker vaccine candidates and vGPE$^-$ in SK-L cells at 37 °C were investigated. In Figure 13, vGPE$^-$/PAPeV E^{rns} exhibited a higher replication tendency compared to vGPE$^-$/PhoPeV E^{rns} but was nearly equivalent to vGPE$^-$, except for higher replication levels than vGPE$^-$ at 1 and 6 dpi. Meanwhile, vGPE$^-$/PhoPeV E^{rns} propagated to nearly the same extent as vGPE$^-$ but at a slower rate than vGPE$^-$ at 1 and 5 dpi. Despite the growth kinetics of the two chimeric viruses being nearly comparable to that of vGPE$^-$, significant differences were observed in IFN-α/β induction when SK-L cells were infected with each virus. Previous studies demonstrated the robust IFN induction capacity of vGPE$^-$ [40,106], which also corroborated with the IFN induction of vGPE$^-$ in this study. Of particular interest is the observation that the IFN-producing capacity of vGPE$^-$/PAPeV E^{rns} surpassed that of vGPE$^-$. A large amount of IFN production by vGPE$^-$/PAPeV E^{rns} persisted from 3 dpi onward. Similarly, vGPE$^-$/PhoPeV E^{rns} exhibited higher IFN induction than vGPE$^-$; however, this discrepancy was not evident at 3 and 4 dpi. Between the two chimeric virus strains, although vGPE$^-$/PhoPeV E^{rns} showed a higher IFN level compared with vGPE$^-$/PAPeV E^{rns} at 1 dpi, significant differences in IFN induction were noted from 2 to 6 dpi, with vGPE$^-$/PAPeV E^{rns} consistently eliciting higher IFN levels than vGPE$^-$/PhoPeV E^{rns}.

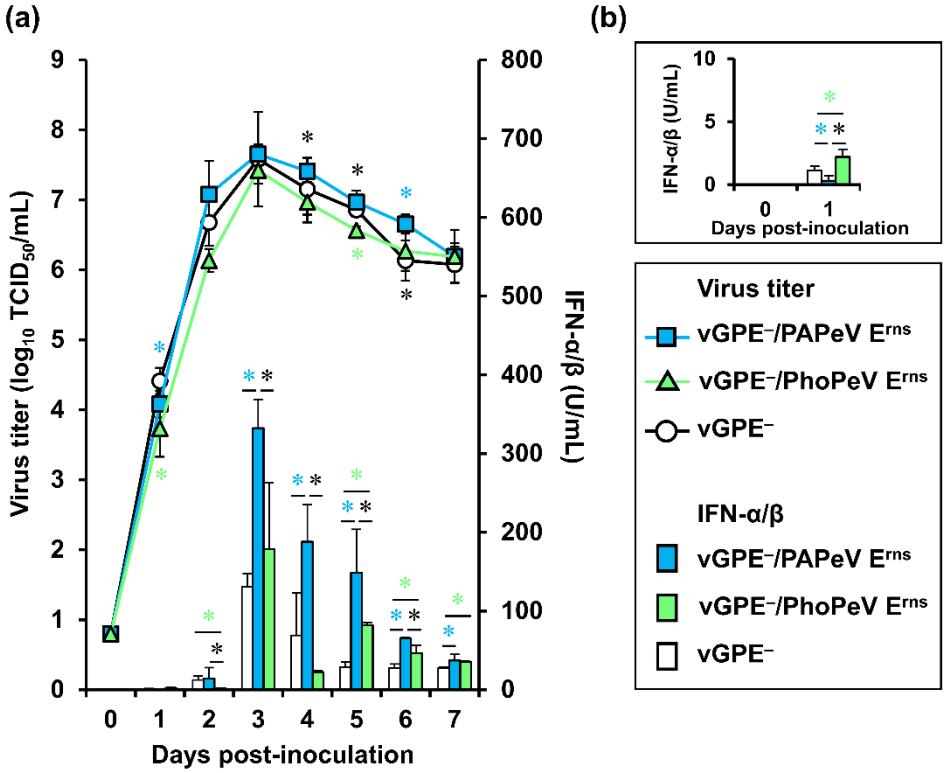


Figure 13. *In vitro* growth kinetics and IFN-α/β induction in SK-L cells inoculated with vGPE⁻/PAPeV E^{rns}, vGPE⁻/PhoPeV E^{rns}, or vGPE⁻. **(a)** SK-L cells were inoculated with vGPE⁻/PAPeV E^{rns}, vGPE⁻/PhoPeV E^{rns}, or vGPE⁻ at a MOI of 0.001 and incubated at 37 °C with 5% CO_2. Cell supernatants were collected at 0, 1, 2, 3, 4, 5, 6, and 7 dpi. Virus titers were measured and displayed as 50% $TCID_{50}$/mL. The IFN-α/β bioactivity in the supernatants of SK-L cells was measured using the SK6-MxLuc cells. **(b)** The bar chart scale depicting IFN-α/β induction at 0 and 1 dpi was enlarged for better visualization and interpretation of data. Error bars represent the SDs. The significance of viral growth differences was indicated. The blue, green, and black asterisks indicate $p < 0.05$ between vGPE⁻ and vGPE⁻/PAPeV E^{rns}, vGPE⁻ and vGPE⁻/PhoPeV E^{rns}, vGPE⁻/PAPeV E^{rns} and vGPE⁻/PhoPeV E^{rns}, respectively.

Discussion

In Japan, the LAV GPE$^-$ strain was developed and extensively utilized to control CSF. The GPE$^-$ strain originated from the highly virulent ALD strain, undergoing multiple passages and biological cloning in swine testicle cells, bovine testicle cells, and primary guinea pig kidney cells [107]. Field studies demonstrated the safety and efficacy of the GPE$^-$ vaccine [61]. Recently, two chimeric candidates, vGPE$^-$/PAPeV E^{rns} and vGPE$^-$/PhoPeV E^{rns}, were synthesized in the joint effort toward marker vaccine development. These candidates were derived from the LAV vGPE$^-$ vaccine strain as a backbone and possessed the glycoprotein E^{rns} sourced from distantly related pestiviruses. Considering the safety assessment according to the previous reports on the virulence gain observed with the GPE$^-$ vaccine strain [37], there arose concerns regarding the utilization of the vGPE$^-$ strain as a backbone and the incorporation of foreign epitopes into LAV strain, which led to questions about their stability and safety profiles. To better understanding these concerns, this study showed that the attenuation of the two chimeric marker vaccine candidates can be affirmed by substituting the CSFV glycoprotein E^{rns} for either PAPeV E^{rns} or PhoPeV E^{rns}. Meanwhile, the vGPE$^-$ strain maintained stable replication without increasing virulence in pigs.

Throughout the serial passages in pigs, the chimeric marker vaccine candidate vGPE$^-$/PAPeV E^{rns} exhibited the highest attenuation level, achieving this within just two passages. Although the recovery of vGPE$^-$/PhoPeV E^{rns} was detectable until the third passage, in which the virus recovery was either below the detection threshold or not present, it completely disappeared by the fourth, affirming its attenuation. The vGPE$^-$ strain, which serves as the backbone for the two chimeric viruses, demonstrated stable virus recovery across all passages without indicating increased virulence in pigs, suggesting potential advantages in terms of safety and efficacy, as confirmed in a previous study [40]. This is attributed to its development through a reverse genetic system-based production method [18,37–39,106,108], ensuring strict quality management for the vaccine. Meanwhile, the changes acquired during passages in pigs inoculated with GPE$^-$ were attributed to the vaccine inoculum harboring a quasispecies population and the experimental design of serial passage in pigs involved administering a high inoculation dose ($10^{7.6}$ $TCID_{50}$) of the GPE$^-$ virus at the first passage, and tonsil homogenates were

then collected at the peak of virus recovery at 4 dpi, as confirmed previously [37], which differed from the design in this study.

The recovered viruses in each passage were utilized to analyze amino acid substitutions in their genomes. Surprisingly, amino acid substitutions in the structural proteins (C and E1) were identified in P1/vGPE^{-}/PAPeV E^{rns}, even though the virus disappeared in the second passage. Given that the last eight residues (262–267) at the C-terminus of the C protein significantly influence the proteasomal degradation of CSFV C protein and determine the cleavage efficiency of the C protein by signal peptide peptidase, as established previously [109], the P264L substitution at the C-terminus of the C protein, observed in the P1/vGPE^{-}/PAPeV E^{rns}, could potentially impact efficient viral replication through interactions with both viral and cellular proteins [110]. The M660T substitution observed in the E1 protein of P1/ vGPE^{-}/PAPeV E^{rns} was found to be identical to the ancestral highly virulent ALD strain of GPE^{-} and vGPE^{-}. Similarly, amino acid homology with the ALD strain was also identified in P2-and P3/ vGPE^{-}/PhoPeV E^{rns} at residue I3842. Among the two amino acid substitutions in the nonstructural protein (NS5B) of P3/vGPE^{-}/PhoPeV E^{rns}, the N3289D substitution was situated in the N-terminal domain (NTD), while another substitution (T3842I) resided in the thumb domain of NS5B. Because the thumb domain within the CSFV NS5B protein had a discriminating role, precisely regulating access to the active site during initiation by restricting entry to the template-binding site [111], the T3842I substitution, which emerged in P2/vGPE^{-}/PhoPeV E^{rns}, and persisted in P3/vGPE^{-}/PhoPeV E^{rns}, was identical to ALD and vALD-A76 strains and might enhance viral replication, which was also found at same position in NS5B of P5/ vGPE^{-}. However, the subsequent discovery of N3289D in the NTD in NS5B protein of P3/vGPE^{-}/PhoPeV E^{rns} raised the hypothesis that the impaired replication observed in P4/vGPE^{-}/PhoPeV E^{rns} could result from disrupting the catalytic activity of the RNA-dependent RNA polymerase [111,112]. Meanwhile, the quasispecies population was identified by the occurrence of amino acid changes in nonstructural proteins (NS2, NS4B, and NS5B) in P5/vGPE^{-}, essential for pestiviral RNA replication [6]. Given the attenuation observed in pigs for both chimeric viruses, the present study did not comprehensively investigate the functional implications of these mutations. However, further studies can elucidate viral attenuation via these amino acid substitutions to understand viral pathogenesis.

Given the attenuation of the two chimeric marker vaccine candidates in pigs, their capacities for IFN-α/β induction were investigated *in vitro*. Pigs inoculated with the highly IFN-α/β-inducing vGPE^{-}/PAPeV E^{rns} strain exhibited viral attenuation within two passages. A similar phenomenon was observed in pigs injected with the IFN-α/β-inducing vGPE^{-}/PhoPeV E^{rns} strain, where the virus became undetectable within four passages. Although the IFN-α/β induction by vGPE^{-} demonstrated a comparable pattern to that of vGPE^{-}/PhoPeV E^{rns} *in vitro*, the virus recovery persisted in tonsils in all pigs until the fifth passage. Previous investigations into the role of E^{rns} as an IFN antagonist primarily focused on evaluating the RNase activity of E^{rns} from BVDV and CSFV, which provided indirect evidence suggesting comparable RNase activities between E^{rns} from BVDV and CSFV [113,114]. A recent study expanded upon this research by assessing the RNase activity of E^{rns} from nearly all viruses classified within the *Pestivirus* genus, including PAPeV E^{rns} and PhoPeV E^{rns} [115]. Notably, the RNase activity of E^{rns} from both PAPeV and PhoPeV was found to be the least active, exhibiting ~20- to 30-fold lower activity than BVDV [115]. It might indirectly support the highly IFN-α/β-inducing vGPE^{-}/PAPeV E^{rns} strain that showed attenuation in pigs in this study. Despite the lack of correlation between the *in vivo* characteristics of vGPE^{-}/PhoPeV E^{rns} and its IFN-α/β induction profile, the mechanism underlying its attenuation in pigs remained elusive. Further research should be performed to elucidate this mechanism comprehensively.

While the current study showed that the two chimeric viruses were attenuated within fewer than five passages, comprehensive studies are still required to fully establish the safety profiles of these chimeric viruses in the future. This includes following the WOAH recommendations [116], such as considering the size and age of the pigs used in the experiments and possibly conducting additional passages in two further piglets once the virus becomes undetectable to assess the stability of the attenuation and the potential for reversion to virulence. Additionally, for practical applications, future studies should prioritize investigating the safety of chimeric viruses in breeding stocks (sows or boars), particularly in pregnant sows, to effectively address the risk of transplacental transmission. This approach will ensure a more accurate and practical safety evaluation, aligning with field conditions and the unique considerations of breeding stocks.

Brief summary

Chimeric marker vaccine candidates, vGPE^{-}/PAPeV E^{rns} and vGPE^{-}/PhoPeV E^{rns}, have been generated and their efficacy and capability to differentiate infected from vaccinated animals were confirmed in previous studies. The safety profile of the two chimeric marker vaccine candidates, particularly in the potential reversion to virulence, was evaluated. Each virus was administered to pigs with a dose equivalent to the vaccination dose, and pooled tonsil homogenates were subsequently inoculated into further pigs. Chimeric virus vGPE^{-}/PAPeV E^{rns} displayed the most substantial attenuation, achieving this within only two passages, whereas vGPE^{-}/PhoPeV E^{rns} was detectable until the third passage but disappeared entirely by the fourth passage. The vGPE^{-} strain, assessed alongside, consistently exhibited stable virus recovery across each passage without any signs of increased virulence in pigs. In conclusion, the safety profile of the two chimeric marker vaccine candidates was affirmed. Further research is essential to ensure the stability of their attenuation and safety in diverse pig populations.

Conclusion

Developing and deploying marker vaccines against CSF represent pivotal advancements in advanced veterinary medicine and livestock management. These marker vaccines play a crucial role in disease control and eradication efforts, particularly by facilitating the DIVA strategies. This capability is essential for international trade, ensuring that vaccinated animals can be distinguished from those carrying the wild-type virus. Marker vaccines also enhance disease surveillance and monitoring in field settings, enabling rapid detection and containment of outbreaks. These vaccines promote sustainable and ethical animal health practices by reducing the economic impact of CSF outbreaks and minimizing the need for mass culling. This study focused on the comprehensive development and assessment of novel chimeric marker vaccine candidates, evaluating their biological profiles and potential application for DIVA strategies in the field.

Chimeric pestivirus offers novel opportunities for developing marker vaccines against CSFV. In this study, the combination of a CSFV LAV strain with the glycoprotein E^{rns}, derived from distantly related pestiviruses, resulted in the development of two chimeric viruses, vGPE^{-}/PAPeV E^{rns} and vGPE^{-}/PhoPeV E^{rns}. These chimeric viruses demonstrated rapid and effective protection against systemic infection by moderately virulent CSFV challenges. The findings underscore the promising potential of these chimeric viruses as marker vaccines, emphasizing their feasibility using the POC diagnostic tools to assess DIVA capabilities directly in field settings.

Building on the exploration of chimeric pestiviruses for marker vaccine development against CSFV, this study further investigated the potential of E2 and E^{rns} antigens derived from plants to engineer a CSFV E2/E^{rns} dual-antibody ICS. This innovative diagnostic tool exhibited a strong correlation with commercial ELISA kits and demonstrated high sensitivity and specificity in detecting E2 and E^{rns} antibodies specific to CSFV. Crucially, the ICS developed in this study aligns with the DIVA concept by utilizing the chimeric vaccine candidates, vGPE^{-}/PAPeV E^{rns} and vGPE^{-}/PhoPeV E^{rns}. These findings underscore its suitability as a rapid diagnostic tool for the surveillance and monitoring of CSFV antibodies in vaccinated pig populations.

In terms of vaccine safety assessment, this study conducted a thorough investigation into the safety profiles of vGPE^{-}/PAPeV E^{rns} and vGPE^{-}/PhoPeV E^{rns} using serial passages in pigs. Although both chimeric viruses demonstrated different degrees of attenuation, their potential suitability as safe vaccine candidates against CSFV was confirmed in this study. However, further studies are still needed to thoroughly determine the safety profiles of these chimeric viruses in adherence to WOAH guidelines. Overall, these findings contribute to refining the understanding of these chimeric vaccines and optimizing their application in CSFV control strategies.

In conclusion, this study highlighted novel strategies in transboundary disease control and eradication, emphasizing the development of promising chimeric viruses vGPE^{-}/PAPeV E^{rns} and vGPE^{-}/PhoPeV E^{rns} as marker vaccine candidates for the control and eradication of CSF. The study comprehensively evaluated their safety, efficacy, and DIVA properties, alongside validating the ICS as a practical diagnostic tool in field settings. These advancements are poised to significantly contribute to the formulation of effective strategies aimed at managing CSFV in pig populations globally, thereby enhancing biosecurity and safeguarding animal health and welfare.

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