

Article

Potency of a Live Attenuated GPE[−] Vaccine Against an Antigenically Distinct Classical Swine Fever Virus Strain in Japan

Tatsuya Nishi ¹, Emiko Ito ², Miyabi Nishimura ², Tomoko Kato ¹, Mizuki Watanabe ¹, Kentaro Masujin ¹, Yoshitaka Imaizumi ² and Katsuhiko Fukai ^{1,*}

- ¹ WOAHP Reference Laboratory for Classical Swine Fever, Kodaira Research Station, National Institute of Animal Health, National Agriculture and Food Research Organization, Kodaira 187-0022, Tokyo, Japan; nishi.tatsuya047@naro.go.jp (T.N.); kato.tomoko243@naro.go.jp (T.K.); watanabe.mizuki595@naro.go.jp (M.W.); masujin.kentaro550@naro.go.jp (K.M.)
- ² Advanced Technology Development Center, Kyoritsu Seiyaku Corporation, 2-9-22 Takamihara, Tsukuba 300-1252, Ibaraki, Japan; e_ito@kyoritsuseiyaku.com (E.I.); m_nishimura@kyoritsuseiyaku.com (M.N.); y_imaizumi@kyoritsuseiyaku.com (Y.I.)
- * Correspondence: fukai.katsuhiko544@naro.go.jp; Tel.: +81-42-321-1441

Abstract

Background: Highly potent vaccines are essential for the effective control of classical swine fever (CSF). Since CSF re-emerged in 2018 in Japan, the live CSF virus (CSFV) vaccine—a guinea pig exaltation of Newcastle disease virus-negative strain vaccine (GPE[−], genotype 1.1)—has been applied to domestic pigs, contributing to a reduction in outbreaks. Meanwhile, the persistence and continued expansion of CSFV in wild boar populations have raised concerns regarding potential antigenic divergence. **Methods:** We systematically evaluated the neutralizing reactivity of sera from GPE[−]-vaccinated pigs against CSFV strains (genotype 2.1) recently circulating in Japan against identified a representative strain that showed markedly reduced neutralization. We directly assessed the protective efficacy of the GPE[−] vaccine against this strain in a controlled challenge experiment. At 4 weeks post-vaccination, both vaccinated and unvaccinated pigs were orally challenged with the representative Japanese strain and monitored for 3 weeks thereafter. **Results:** Among the Japanese CSFV strains, the JPN/SM/WB/2022 isolate exhibited markedly reduced neutralizing reactivity—over 32-fold lower than that against the vaccine strain—when tested with GPE[−] vaccine-induced antisera. In the experimental infection in pigs, unvaccinated pigs exhibited typical clinical signs of CSF and viremia, and two pigs reached the humane endpoint. In contrast, none of the vaccinated pigs showed any clinical signs of infection. Robust humoral and cellular immune responses were induced in vaccinated pigs, which may correlate with the observed complete protection. **Conclusions:** The GPE[−] live vaccine provides protective immunity against an antigenically distinct strain, prevents disease, and limits viral spread in domestic pigs.

Keywords: classical swine fever; GPE[−] strain; live attenuated vaccine; antigenic divergence



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1. Introduction

Classical swine fever (CSF) is a highly contagious viral disease that causes severe economic losses due to high mortality and trade restrictions following outbreak notifications [1,2]. Classical swine fever virus (CSFV) is a positive-sense RNA virus belonging to the genus *Pestivirus* in the family *Flaviviridae* [3]. After entry, CSFV replicates initially in

the tonsils and then spreads through the lymphatic system to regional lymph nodes; this is followed by viremia and dissemination to secondary target organs, such as the spleen, bone marrow, intestinal lymphoid tissues, and parenchymatous organs at later stages [1,4]. CSFV strains exhibit a wide range of virulence profiles, from highly virulent strains that cause nearly 100% mortality to moderately virulent or avirulent strains [1,2].

CSFV is classified into three major genotypes (1, 2, and 3), with additional genotypes (4 and 5) recently proposed, and multiple subgenotypes including 1.1–1.4, 2.1–2.3, and 3.1–3.4 [1,2,5]. Genotype 2.1 is currently the most prevalent and genetically diverse lineage globally, and is antigenically distinct from the commonly used genotype 1.1 vaccine strains [1,2,5,6]. Numerous previous studies have demonstrated that genotype 2.1 strains are generally moderately virulent, which can hamper prompt clinical recognition and notification in the field [7–11]. Notably, since late 2014, genotype 2.1d strains have caused outbreaks even in pig farms routinely vaccinated with C-strain vaccines, raising concerns about potential antigenic drift and incomplete vaccine protection [12]. Amino acid variations driven by positive selection have been documented in the antigenic epitopes of the E2 protein in 2.1d strains, suggesting ongoing antigenic variation [13]. Evaluations of commercial C-strain-based vaccines have shown that vaccine-induced antisera exhibit significantly lower neutralizing titers against subgenotype 2.1d isolates [14]. Although C-strain vaccination protected pigs from clinical disease following challenge with a representative 2.1d isolate, mild pathological lesions and detectable viral RNA were observed in multiple tissues and blood samples, indicating incomplete virological protection [14]. These findings highlight that antigenic differences among genotype 2.1d viruses may have biological relevance, underscoring the importance of evaluating vaccine efficacy against antigenically divergent field strains.

CSF re-emerged in Japan in September 2018, ending a 26-year period of absence, following the detection of JPN/1/2018, the first reported subgenotype 2.1d isolate in the country [15]. Experimental studies have confirmed that recent Japanese isolates are moderately virulent, but highly transmissible to domestic pigs and wild boars [16]. Despite intensive control measures, including stamping out and vaccination of domestic pigs, as well as oral vaccination of wild boars, CSFV has spread to 100 domestic pig farms, and more than 9000 infected wild boars have been identified as of November 2025 [17].

The live attenuated guinea pig exaltation-negative (GPE[−]) vaccine has been widely implemented as a practical tool for CSF prevention in Japan and in several countries in Southeast Asia. High protective efficacy of the GPE[−] live vaccine against Japanese epidemic strains, including genotype 2.1 strains, has been reported [18,19]. Nevertheless, the persistence and continued expansion of genotype 2.1d viruses in wild boar populations have raised concerns regarding potential antigenic divergence from the vaccine strain. Under these circumstances, determining whether the currently circulating strains exhibit antigenic variation and verifying its impact on vaccine-induced protection are crucial for reinforcing evidence-based control strategies.

In this study, we systematically evaluated the neutralizing reactivity of GPE[−] vaccine-induced antisera against recent Japanese CSFV isolates and identified a representative genotype 2.1d strain that showed markedly reduced neutralization. We directly assessed the protective efficacy of the long-established GPE[−] live attenuated vaccine against this strain in a controlled challenge experiment, thereby providing evidence to clarify the relationship between antigenic variations and vaccine-induced protection.

2. Materials and Methods

2.1. Cells and Viruses

The porcine kidney-derived cell line (CPK) was cultured in Dulbecco's modified Eagle's medium/Nutrient Mixture F12 (DMEM/F-12; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% pestivirus-free fetal calf serum (Japan Bio Serum Co., Ltd., Tokyo, Japan). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂.

CSFV strains isolated in Japan between 2018 and 2022 (Supplementary Table S1) were recovered and propagated in CPK cells. Virus stocks were aliquoted and stored at −80 °C until use. CSFV JPN/WB_SM325/2022 (JPN/SM/WB/2022) was isolated from serum samples collected from wild boars captured in Shimane Prefecture, Japan, in 2022.

2.2. CSFV Vaccines

Two live attenuated CSFV vaccines derived from the GPE[−] strain, which represent the current licensed standards for CSF vaccination in Japan, were used in this study. Swivac C (Kyoritsu Seiyaku Corporation, Tokyo, Japan) was administered at a dose of 1 mL per pig, whereas Swivac C 0.5 (Kyoritsu Seiyaku) was administered at a dose of 0.5 mL per pig, in accordance with the manufacturer's approved instructions. Swivac C is required to contain a viral potency of ≥ 3.0 TCID₅₀/mL, and Swivac C 0.5 is required to contain ≥ 3.3 TCID₅₀/mL, in compliance with the national regulatory standards for veterinary biological products in Japan. The vaccines used in this study met these official potency requirements and were confirmed to contain equivalent antigen content. The vaccines were stored and handled at temperatures not exceeding 10 °C in accordance with the manufacturer's recommendations until use.

2.3. Neutralization Testing with GPE[−] Vaccine-Induced Antisera

Two 4-week-old crossbred Landrace × Large White × Duroc pigs were vaccinated with Swivac C (Kyoritsu Seiyaku). Sera were collected from the pigs at 6 weeks post-vaccination. Viral neutralization tests for each strain were performed according to the WOAHE Terrestrial Manual of 2022 [20]. Sera were heated at 56 °C for 30 min to inactivate the complement system in the serum samples. The heat-inactivated sera were diluted two-fold and mixed with 200 TCID₅₀ of the GPE[−] and CSFV strains isolated in Japan from 2018 to 2022 (listed in Supplementary Table S1). After incubation at 37 °C for 1 h, the serum–virus mixtures were transferred to 96-well plates containing CPK cell monolayers and incubated at 37 °C with 5% CO₂ for 4 days. The cells were subsequently fixed with 80% acetone. For virus detection, a CSFV anti-E2 monoclonal antibody (WH303; Animal and Plant Health Agency, Surrey, UK) was used as the primary antibody, followed by a goat anti-mouse immunoglobulin G (H+L) cross-absorbed secondary antibody conjugated with Alexa Fluor 488 (Thermo Fisher Scientific). Fluorescence signals were observed using an LSM700 confocal microscope (Zeiss, Oberkochen, Land Baden-Württemberg, Germany). Virus neutralization titers (VNTs) were determined as the reciprocal of the highest serum dilution that inhibited viral growth in at least one of the two replicate wells. VNTs were measured in triplicate, and mean values were calculated. For statistical comparison of neutralizing antibody titers between the JPN/1/2018 and JPN/SM/WB/2022 isolates, a two-tailed Welch's t-test (unequal variance t-test) was performed using Microsoft Excel. Technical triplicate measurements from two vaccinated pigs were used as individual data points ($n = 6$ per group). A p -value < 0.05 was considered statistically significant.

2.4. Experimental Infections

Fifteen 4-week-old crossbred Landrace × Large White × Duroc pigs were used in this study to reflect standard field practice, as CSF vaccination is routinely administered shortly after weaning in Japan and many other countries, and this age is consistent with WOAHP recommendations for evaluating live attenuated CSF vaccines [20]. Before the experiments, the pigs were confirmed to lack antibodies against CSFV. Pigs were randomly allocated to three groups. Group 1 (Nos. 1–5) was vaccinated with Swivac C (Kyoritsu Seiyaku), and Group 2 (Nos. 6–10) was vaccinated with Swivac C 0.5 (Kyoritsu Seiyaku). Group 3 (Nos. 11–15) served as the non-vaccinated control group. After vaccination, serum samples were collected weekly. At 28 days post-vaccination (dpv), all pigs were orally inoculated with 2 mL of $10^{5.0}$ TCID₅₀ JPN/SM/WB/2022 suspended in DMEM/F-12. The oral route was selected to mimic the predominant natural route of CSFV transmission. This inoculation route and dose have been shown to reliably infect pigs with the JPN/1/2018 strain and to approximate natural infection conditions, as previously reported [16]. Following virus inoculation, clinical samples, including whole blood, serum, and oral swabs, were collected at 3-day intervals until 18 days post-inoculation (dpi). Oral swabs were suspended in ten volumes of DMEM/F-12 and clarified using a 0.45 µm centrifugal filter unit (Ultrafree-MC; Merck Millipore, Darmstadt, Germany). Total leukocyte counts in whole blood were measured using an automated hematology analyzer (Celltac α MEK-6558; Nihon Kohden, Tokyo, Japan). Pigs that survived the experimental period were euthanized under deep anesthesia induced by ketamine injection (Daiichi Sankyo Propharma, Tokyo, Japan), followed by humane exsanguination via the axillary artery. Necropsy was performed at 21 dpi (Groups 1–2) and 22 dpi (Group 3).

2.5. Ethics

All animal experiments were reviewed and approved by the Animal Care and Use Committee of the National Institute of Animal Health (NIAH) prior to study initiation (authorization numbers: 20-036 and 24-027). Experimental infection with JPN/SM/WB/2022 was conducted in a high-containment facility at NIAH. This facility met the containment requirements for Group 4 pathogens as specified in the WOAHP Terrestrial Manual [21]. During the experiments, the animals were monitored daily by trained veterinarians for body temperature, behavior, and clinical condition.

2.6. Isolation of Porcine Peripheral Blood Mononuclear Cells (PBMCs)

Whole blood was collected from all pigs at 28 days post-vaccination (dpv) using Venoject II VP-NA070K tubes (Terumo, Tokyo, Japan). Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation with Ficoll-Paque PLUS (Cytiva, Marlborough, MA, USA). Isolated PBMCs were resuspended in DMEM (Nacalai Tesque, Kyoto, Japan) supplemented with 10% (*v/v*) fetal bovine serum (Japan Bio Serum) and 1% (*v/v*) penicillin–streptomycin solution (10,000 U/mL penicillin and 10 mg/mL streptomycin; Nacalai Tesque).

2.7. In Vitro Stimulation Assay for Detection of CSFV-Specific Interferon-γ by Enzyme-Linked Immunosorbent Assay (ELISA)

An in vitro PBMC stimulation assay was conducted as previously described [22], with minor modifications. PBMCs were enumerated and adjusted to a concentration of 2×10^5 cells per 100 µL. Aliquots of 100 µL were dispensed into each well of a 96-well U-bottom plate. Cells were stimulated by adding 100 µL of medium containing either the GPE[−] or JPN/SM/WB/2022 strain at a multiplicity of infection (MOI) of 1. As a negative control, a mock inoculum prepared from uninfected CPK cell supernatant was added to an equivalent volume. After incubation for 72 h at 37 °C in a humidified 5%

CO₂ atmosphere, culture supernatants were collected and stored at −80 °C until analysis. The concentration of porcine IFN-γ in supernatants was measured using the ELISA Flex: Porcine IFN-γ (HRP) kit (Mabtech AB, Nacka Strand, Sweden), according to the manufacturer's instructions. Differences in IFN-γ responses of PBMCs stimulated with the GPE[−] strain or JPN/SM/WB/2022 were evaluated using two-tailed paired Student's t-test (Microsoft Excel, t-Test: Paired Two Sample for Means). Normal distribution of paired differences was assumed, as required for the paired t-test. A *p*-value < 0.05 was considered statistically significant.

2.8. Tissue Samples

During necropsy of experimentally infected pigs, tissue samples from the tonsils, spleen, kidneys, and ears were collected immediately after confirmation of death (within 15 min), placed in sterile Petri dishes, and kept on ice until further processing. Tissue homogenate samples were prepared using a Micro Smash MS-100R (Tomy Seiko, Tokyo, Japan) equipped with a cooling function, and homogenization was performed under refrigerated conditions. The homogenates were emulsified in DMEM/F12 to yield 10% (*w/v*) suspension and centrifuged at 8000 × *g* for 10 min to collect a clear supernatant. The supernatants were filtered using a 0.45 μm centrifugal filter unit (Ultrafree-MC, Merck Millipore, Darmstadt, Germany) before viral titration.

2.9. Viral Titration and Neutralization Tests

Viral titration and virus neutralization tests were carried out for each strain in accordance with the WOAHP Terrestrial Manual (2022) [20]. For viral titration, ten-fold serial dilutions of clinical samples were prepared and inoculated into quadruplicate wells of 96-well plates seeded with CPK cells. The plates were incubated at 37 °C in a 5% CO₂ atmosphere for 4 days, after which they were fixed in ice-cold 99.9% ethanol. Similar to the neutralization tests described above, primary and secondary antibody reactions were performed, and the results were recorded using an LSM700 (Zeiss). Viral titers were determined using the Behrens–Kärber method.

For virus neutralization tests, serum samples were mixed with an equal volume of chloroform to eliminate residual infectious CSFV, followed by centrifugation at 14,000 × *g* for 10 min at 4 °C. The recovered supernatants were heat-treated at 56 °C for 30 min to inactivate complement. Heat-inactivated sera were then serially diluted two-fold and combined with 200 TCID₅₀ of the GPE[−] or JPN/SM/WB/2022 strains. After incubation at 37 °C for 1 h, the mixtures were added to CPK cell monolayers in 96-well plates and incubated for 4 days under standard culture conditions. Viral growth was assessed using the same immunostaining procedure as described for viral titration. Neutralizing antibody titers were expressed as the reciprocal of the highest serum dilution that inhibited virus replication in at least one of two replicate wells.

2.10. Real-Time Reverse Transcription-Polymerase Chain Reaction (rRT-PCR)

Viral nucleic acids were extracted from clinical samples (whole blood, serum, and oral swabs) and tissue samples (tonsils, spleens, kidneys, and ears) using Solution N and Tissue Direct Solution E (Takara Bio, Kusatsu, Shiga, Japan), respectively, according to the manufacturer's instructions. These reagents have been used to rapidly prepare crude nucleic acid samples.

CSFV-specific genes were detected with rRT-PCR by using the CSFV/ASFV Direct RT-qPCR Mix & Primer/Probe Ver. 2 (Takara Bio) [23] according to the manufacturer's instructions. Swine GAPDH-specific genes were simultaneously detected as internal controls to verify the efficiency of nucleic acid extraction and the accuracy of the rRT-PCR assay (Supplementary Table S3). Among the CSFV-positive samples, tonsil samples

derived from vaccinated pigs were further tested to determine whether the detected gene originated from the vaccine or challenge strain. Differentiation was conducted using CSFV (Genotype 1) Direct RT-qPCR Mix & Primer/Probe (Takara Bio Inc.), which specifically detects CSFV genotype 1. All multiplex RT-PCR assays were performed using the Applied Biosystems QuantStudio 5 Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). Any detectable amplification signal was interpreted as positive, and therefore no specific C_t threshold was applied.

2.11. CSFV E2-Specific Antibody ELISA

Anti-CSFV antibodies in serum samples collected from the pigs were examined using a CSFV E2-specific ELISA kit, a Classical Swine Fever ELISA kit II (Nippon Gene, Toyama, Japan), and the IDEXX CSFV Ab Test (IDEXX Laboratories, Westbrook, ME, USA), following the manufacturer's instructions.

3. Results

3.1. Neutralizing Reactivity of GPE^- Vaccine-Induced Antisera Against Japanese Epidemic CSFV Strains

To evaluate the neutralizing reactivity of GPE^- vaccine sera against circulating CSFV strains in Japan, 34 CSFV isolates obtained from domestic pigs and wild boars between 2018 and 2022 were tested in a virus neutralization test (Figure 1). Neutralizing reactivity was detected against all tested isolates, although VNTs varied among the strains. The mean VNT of sera from two vaccinated pigs was $2^{9.4}$ against the homologous vaccine strain (GPE^- , genotype 1.1). Against JPN/1/2018 (genotype 2.1), the index strain of the 2018 outbreak, the mean VNT was $2^{7.4}$. Among the Japanese isolates tested, the lowest neutralizing reactivity was observed against JPN/SM/WB/2022 (genotype 2.1) with a mean VNT of $2^{4.4}$. Statistical comparison demonstrated that the neutralizing reactivity against JPN/SM/WB/2022 was significantly lower than that against JPN/1/2018 ($p = 0.002$), confirming a meaningful antigenic difference between these two contemporary Japanese isolates. To assess the biological relevance of the reduced neutralizing reactivity observed in JPN/SM/WB/2022, this isolate was selected for subsequent challenge experiments in GPE^- -vaccinated pigs.

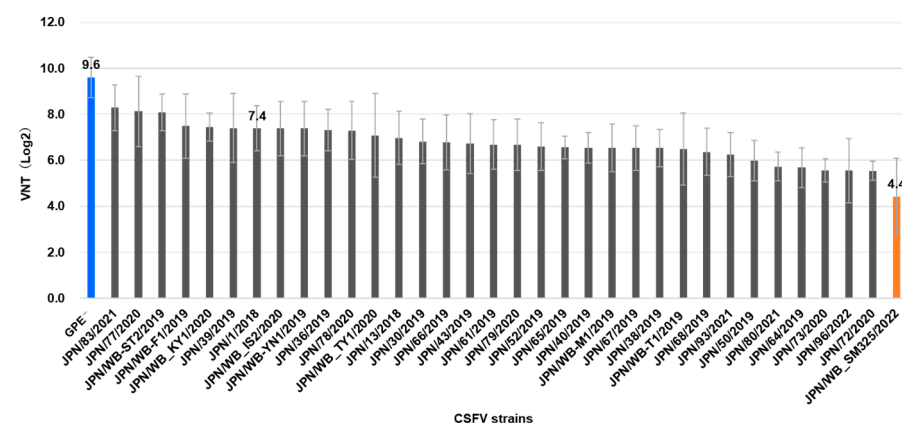


Figure 1. Neutralizing reactivity of GPE^- vaccine-induced antisera against Japanese epidemic CSFV strains. Virus neutralization titers (VNTs) of GPE^- vaccine sera against the vaccine strain and CSFV isolates collected in Japan between 2018 and 2022. The y-axis shows VNTs in $\log_2$ scale. The homologous vaccine strain (GPE^-) is shown in blue, and the challenge strain JPN/SM/WB/2022 is shown in orange. Data are shown as mean $\pm$ standard deviation. Details of the tested viral isolates are provided in Supplementary Table S1.

3.2. Induction of Humoral and Cellular Immunity in GPE[−]-Vaccinated Pigs

In Groups 1 and 2 (Nos. 1–10), which were vaccinated with Swivac C and Swivac C 0.5, respectively, humoral and cellular immunity developed before viral challenge. VNTs against the homologous GPE[−] strain were detected in all pigs from 14 dpv and ranged from 16 to 64 on the day of challenge (28 dpv; 0 dpi) (Figure 2A). In contrast, VNTs against JPN/SM/WB/2022 were detected in all pigs at 21 or 28 dpv; however, their levels remained substantially lower, ranging from 2 to 8, with only two pigs (Nos. 3 and 9) showing higher titers of 32 against this heterologous strain. These results indicate a marked reduction in the cross-neutralizing reactivity of vaccine-induced sera against JPN/SM/WB/2022 compared to the vaccine strain.

To assess cellular immunity, IFN- γ production by PBMCs stimulated with CSFV GPE[−] and JPN/SM/WB/2022 antigens was evaluated in three pigs per group. All vaccinated pigs exhibited clear IFN- γ induction in response to both antigens. However, IFN- γ responses to JPN/SM/WB/2022 were significantly lower than those induced by GPE[−] stimulation (two-tailed paired Student's *t*-test, *t* = 3.32, *df* = 5, *p* = 0.021). In contrast, PBMCs from non-vaccinated control pigs showed negligible IFN- γ responses to either antigen (Figure 2B).

These data demonstrate that GPE[−] vaccination elicited both humoral and cellular immune responses before viral challenge, whereas the magnitude of both responses was reduced against the JPN/SM/WB/2022 strain; these results are consistent with antigenic differences between the vaccine strain and field isolate.

3.3. Clinical Signs in GPE[−]-Vaccinated Pigs Challenged with the JPN/SM/WB/2022

After challenge with JPN/SM/WB/2022, all non-vaccinated control pigs (Nos. 11–15) developed pyrexia at 4 dpi and leukopenia at 3 dpi (Figure 3). Conjunctivitis was observed in pig Nos. 11–13 and 15 from 9 dpi, and all five pigs showed reduced appetite after 14 dpi. Pig Nos. 13 and 15 developed cyanosis at 12 dpi, exhibited severe depression with recumbency (reaching the humane endpoint), and were euthanized at 17 and 16 dpi, respectively (Supplementary Figure S1).

In Groups 1 and 2 (Nos. 1–10) subjected to GPE[−] vaccination, none of the pigs developed clinical signs after viral challenge. Although two pigs (Nos. 2 and 10) showed persistently low white blood cell counts, these values did not decline compared with their baseline levels (Figure 3).

3.4. Virus Detection in GPE[−]-Vaccinated Pigs Challenged with the JPN/SM/WB/2022

In non-vaccinated control pigs (Nos. 11–15), viral titrations of whole blood and oral swabs confirmed infectious CSFVs at 6–21 and 3–21 dpi, ranging from $10^{1.75}$ to $10^{4.50}$ and $10^{1.75}$ to $10^{4.00}$ TCID₅₀/50 μ L, respectively (Figure 4). CSFV RNAs were detected in whole blood, serum, and oral swabs at 3 dpi, while also being detected in all samples until the end of the experimental period (Supplementary Table S2).

In vaccinated pigs, i.e., Groups 1 and 2 (Nos. 1–10), an infectious virus was detected only in the whole blood of pig No. 5 at 6 dpi at a low titer ($10^{0.75}$ TCID₅₀/50 μ L) (Figure 4). Consistently, CSFV RNA was detected only in the same blood sample from pig No. 5 at 6 dpi, whereas transient CSFV RNA detection was also noted in oral swabs from pig Nos. 5–7 at 3 or 6 dpi (Supplementary Table S2). No other clinical samples from the vaccinated pigs tested positive for infectious viruses or viral RNA. These data indicate that viral replication and shedding are limited in vaccinated pigs.

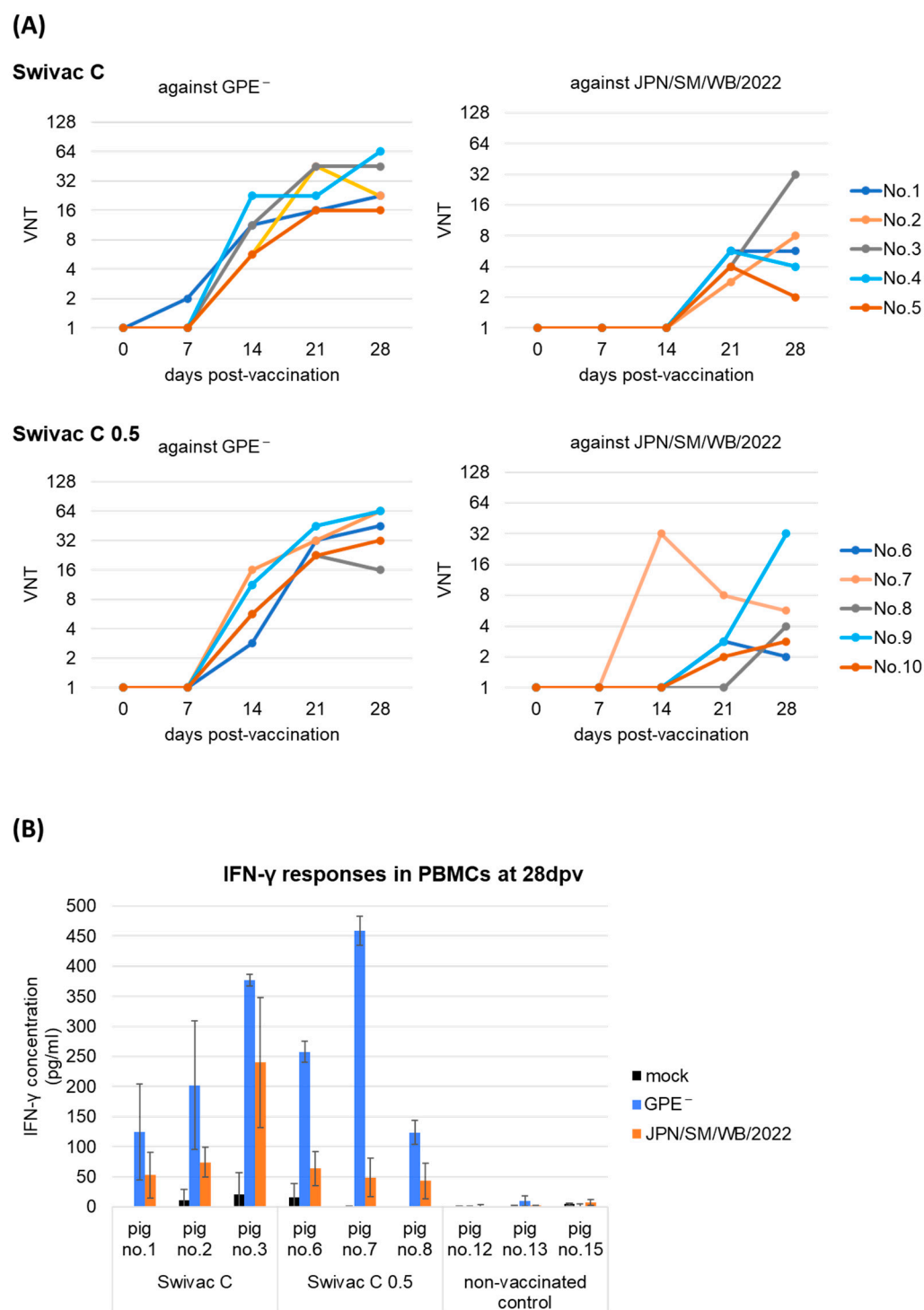


Figure 2. Induction of humoral and cellular immunity in vaccinated pigs. **(A)** Dynamics of neutralizing antibody titers in pigs before and after virus challenge. Virus neutralization titers (VNTs) against the GPE⁻ strain (upper panel) and JPN/SM/WB/2022 (lower panel) are shown in log₂ scale. Titters were measured once a week after vaccination. The x-axis shows the number of days post-vaccination (dpv). **(B)** IFN- γ responses in PBMCs before virus challenge. Peripheral blood mononuclear cells (PBMCs) were collected from three pigs per group at 28 dpv; these cells stimulated with the GPE⁻ or JPN/SM/WB/2022 at a multiplicity of infection (MOI) of 1 for 72 h or mock-treated, and IFN- γ concentrations in culture supernatants were quantified using ELISA. Data are shown as mean $\pm$ standard deviation.

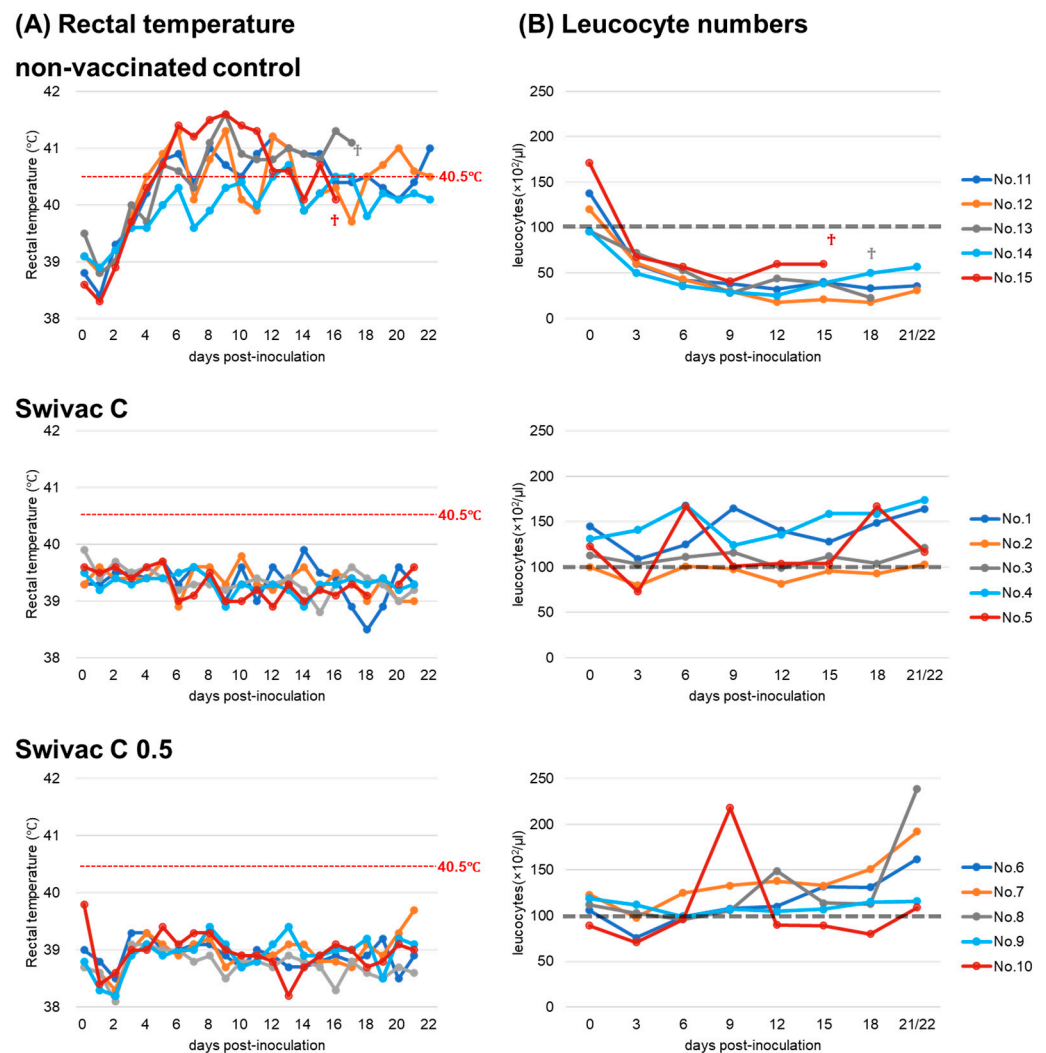


Figure 3. Rectal temperature and leukocyte numbers in pigs after virus challenge. The x-axis shows days post-inoculation (dpi). Pig Nos. 13 and 15 reached the humane endpoint and were euthanized at 17 and 16 dpi, respectively. (A) Daily rectal temperatures after challenge with JPN/SM/WB/2022. (B) Total white blood cell counts in whole blood measured every 3 days post-challenge. † indicates animals that reached humane endpoint.

3.5. Pathological Findings and Virus Distribution in Pigs at the End of the Challenge Experiment

In the non-vaccinated control pigs, necropsy revealed typical CSF-associated gross lesions, including pleural effusion, ascites, petechiae in the kidneys, and enlargement of the mesocolic and inguinal lymph nodes (Supplementary Figure S2). CSFV RNAs were detected in the tonsils, spleen, kidneys, and ear skin (Table 1). Infectious viruses were isolated from these tissues at titers ranging from $10^{2.50}$ to $10^{3.50}$, $10^{3.50}$ to $10^{4.00}$, $10^{3.25}$ to $10^{4.25}$, and $10^{2.75}$ to $10^{3.50}$ TCID₅₀/50 μL, respectively. In the GPE[−]-vaccinated pigs (Nos. 1–10), CSFV RNAs were detected only in the tonsils (Table 1). Genotype-specific rRT-PCR analysis revealed that the RNA detected in pigs Nos. 3, 8, and 9 was CSFV genotype 1, indicating vaccine-derived genes; meanwhile, RNA in pigs No. 7 and 10 was not of genotype 1, suggesting the presence of challenge virus-derived genes. However, no infectious viruses were isolated from any vaccinated pigs. These results indicate that viral replication and dissemination were effectively suppressed in GPE[−]-vaccinated pigs.

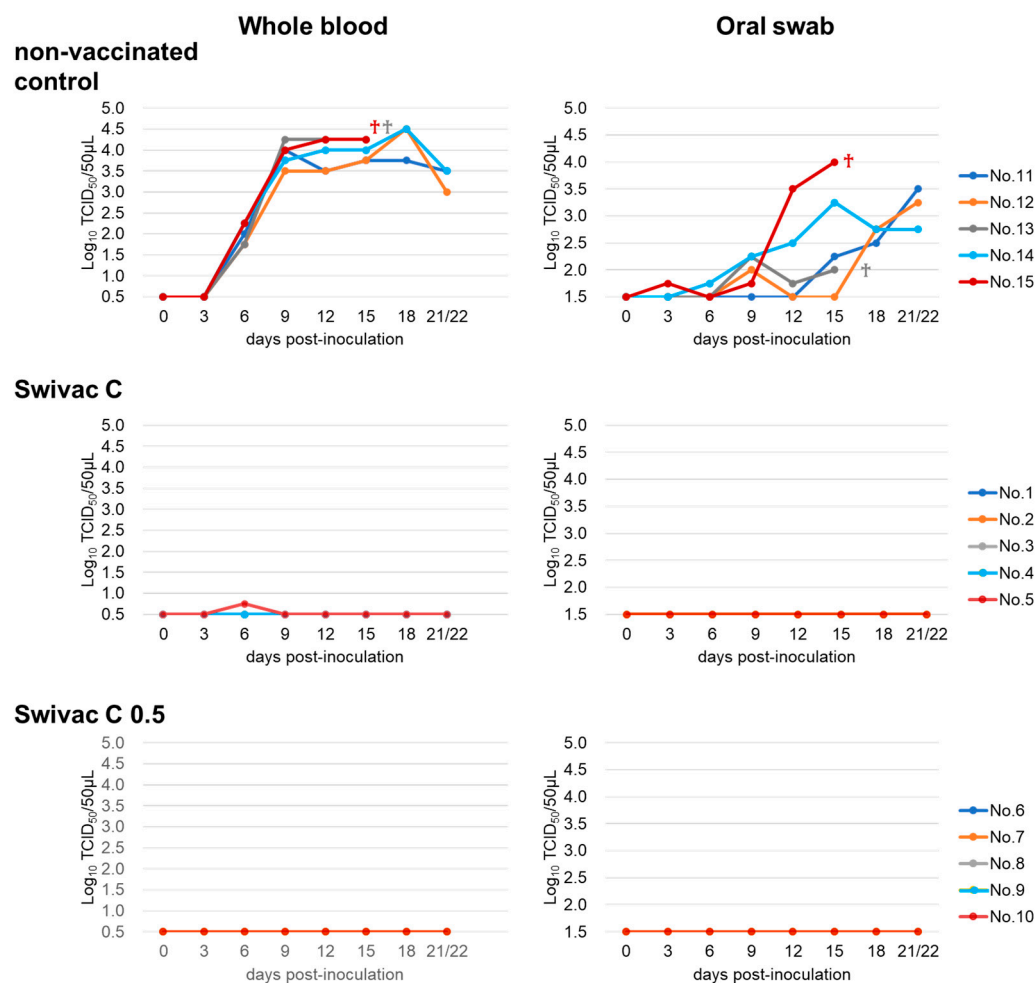


Figure 4. Viral titers in whole blood and oral swab samples collected from pigs following virus challenge. The x-axis indicates the number of days post-inoculation (dpi), while the y-axis represents viral titers expressed as $\log_{10}$ TCID₅₀ per 50 μ L. Whole blood and oral swab samples were obtained at three-day intervals after virus challenge. The detection limit for viral titration in both whole blood and oral swab samples was $<10^{0.5}$ TCID₅₀/50 μ L and $<10^{1.5}$ TCID₅₀/50 μ L, respectively. TCID₅₀: Tissue culture infection dose. † indicates animals that reached humane endpoint.

Table 1. Virus distribution in pigs at the end of the challenge experiment.

Group	Pig No.	dpi	Tonsil		Spleen		Kidney		Ear	
			CSFV RNA ^{a,b}	Viral Titer ^c	CSFV RNA	Viral Titer	CSFV RNA	Viral Titer	CSFV RNA	Viral Titer
Group 1 Swivac C-vaccinated	1	21	—	—	—	—	—	—	—	—
	2	21	—	—	—	—	—	—	—	—
	3	21	32.95 _{gen 1}	—	—	—	—	—	—	—
	4	21	—	—	—	—	—	—	—	—
	5	21	—	—	—	—	—	—	—	—
Group 2 Swivac C 0.5-vaccinated	6	21	—	—	—	—	—	—	—	—
	7	21	35.71	—	—	—	—	—	—	—
	8	21	34.13 _{gen 1}	—	—	—	—	—	—	—
	9	21	34.36 _{gen 1}	—	—	—	—	—	—	—
	10	21	36.12	—	—	—	—	—	—	—

Table 1. Cont.

Group	Pig No.	dpi	Tonsil		Spleen		Kidney		Ear	
			CSFV RNA ^{a,b}	Viral Titer ^c	CSFV RNA	Viral Titer	CSFV RNA	Viral Titer	CSFV RNA	Viral Titer
Group 3 Non-vaccinated control	11	22	21.93	3.00	22.33	4.00	23.03	4.00	24.10	3.00
	12	22	21.61	2.50	22.57	3.75	24.40	3.25	24.63	3.00
	13	17	21.24	3.50	22.38	3.50	24.84	3.75	23.83	2.75
	14	22	21.91	3.00	22.26	3.75	23.80	3.75	24.19	2.75
	15	16	21.61	2.75	21.66	4.00	24.96	4.25	24.03	3.50

^a Ct values of real-time RT-PCR for the detection of CSFV-specific genes were indicated. Cases that were Ct value < 28 are shaded light gray. —, not detected. ^b gen 1, positive for real-time RT-PCR for CSFV Genotype 1, to which GPE[−] vaccine is classified. ^c Viral titers of 10% tissue homogenates were indicated as 10^x TCID₅₀/50 µL. Cases with infectious virus titers are shaded dark gray. —, below the detection limit (10^{0.5} TCID₅₀/50 µL).

3.6. Antibody Responses in Pigs After the Challenge

In non-vaccinated control pigs, the VNT levels remained below the detection limit (<1) until 12 dpi (Figure 5). Low neutralizing activity was detected with VNTs of 2–8 against JPN/SM/WB/2022 at 12 and 15 dpi (pigs Nos. 12 and 13) and a VNT of 2 against GPE[−] at 15 dpi (pig No. 13). CSFV-specific antibodies were detected using ELISA in three control pigs (Nos. 12–14) between 12 and 22 dpi (Supplementary Table S4).

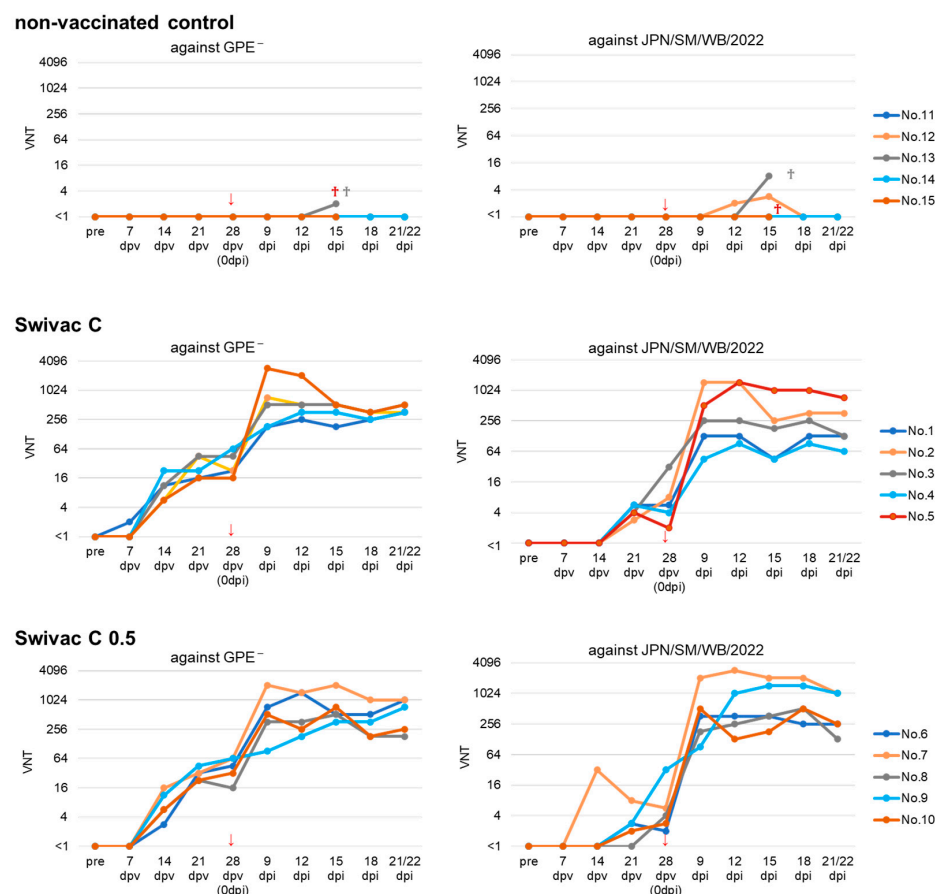


Figure 5. Dynamics of neutralizing antibody titers in pigs before and after virus challenge. Virus Neutralization titers (VNTs) against the GPE[−] strain (upper panel) and JPN/SM/WB/2022 (lower panel) are shown in log₂ scale. Titers were measured once weekly during the vaccination phase and every 3 days after 9 days post-inoculation (dpi). Viral challenge was conducted 28 days post-vaccination (dpv), as indicated by the red arrow. The x-axis shows the number of days post-inoculation. Pigs Nos. 13 and 15 reached the humane endpoint and were euthanized at 17 and 16 dpi, respectively. † indicates animals that reached humane endpoint.

In contrast, vaccinated pigs exhibited a rapid and marked increase in VNT levels after the challenge experiment. Notably, VNTs increased to similarly high levels against both the homologous GPE[−] strain and the heterologous JPN/SM/WB/2022 strain, ranging from 90 to 2896 and from 45 to 2896, respectively; these levels were maintained throughout the observation period (Figure 5). CSFV-specific antibodies were also continuously detected by using ELISA in the vaccinated pigs after the challenge (Supplementary Table S4).

4. Discussion

In the present study, we identified a genotype 2.1d CSFV strain with markedly reduced neutralizing reactivity to GPE[−] vaccine-induced antisera. Subsequently, we evaluated vaccine efficacy against this antigenically distinct strain in a controlled challenge experiment. Using two live attenuated GPE[−]-derived vaccines, which represent the current licensed standards for CSF vaccination in Japan, we demonstrated that the vaccinated pigs exhibited neither clinical signs nor detectable viremia following challenge; therefore, both vaccines conferred complete clinical protection with no apparent difference. These findings indicate that the GPE[−] vaccine provides strong protection even against antigenically divergent strains.

A recent challenge study also reported the high protective efficacy of the GPE[−] vaccine against a Japanese epidemic genotype 2.1 strain, as well as protection induced by a CP7_E2alf marker vaccine [19]. Our results are consistent with these findings and extend them by demonstrating protection against currently circulating field viruses.

Previous studies have reported the emergence of genotype 2.1d strains in C-strain-vaccinated pig farms, incomplete virological protection conferred by C-strain-based vaccines, and amino acid substitutions under positive selection within major antigenic epitopes of the E2 protein [12–14]. In particular, amino acid residues at positions 17, 34, 72, and 200 in the E2 region have been suggested to contribute to antigenic variation. Japanese epidemic genotype 2.1d strains examined in the present study, including JPN/SM/WB/2022, shared the same amino acids at these positions as those previously reported to be associated with antigenic variation. In addition, sequence comparison revealed several amino acid differences between JPN/SM/WB/2022 and other recent Japanese genotype 2.1d strains, such as JPN/1/2018; however, the relevance of these substitutions to the observed antigenic differences requires further investigation. Despite these antigenic differences, viral replication in vaccinated pigs was extremely limited in the present challenge experiments. Infectious viruses were isolated only once from a single vaccinated pig at a low titer, and viral RNA detection was transient and restricted to a small number of samples. Moreover, no infectious virus was detected in any tissues collected at necropsy, indicating that GPE[−] vaccine-induced immunity effectively suppressed viral replication and dissemination in pigs. These findings suggest that, even with amino acid variations within genotype 2.1d CSFV strains, such differences did not result in immune escape under the experimental conditions of this study.

Although both VNTs and IFN- γ responses against the heterologous strain were markedly lower than those against the vaccine strain before challenge, a rapid and pronounced increase in neutralizing titers against the antigenically distinct challenge strain was observed following infection, reaching levels comparable to those against the homologous strain. This rapid increase in neutralizing antibody titers, together with vaccine-induced cellular immunity, likely contributed to the complete protection observed in vaccinated pigs and highlighted the high protective efficacy of the live attenuated GPE[−] vaccine. The ability of the GPE[−] live vaccine to induce both humoral and cellular immunity may explain its resilience against antigenic variation among circulating field strains.

Live attenuated CSFV vaccines have been reported to induce strong virus-specific IFN- γ -producing T-cell responses, particularly CD8⁺ T cells, which can play an important role in controlling viral replication even before the development of high neutralizing antibody titers [24–26]. Such cellular immunity may provide cross-strain protection by recognizing conserved viral epitopes that are not directly targeted by neutralizing antibodies [22]. Furthermore, as primary CSFV replication occurs in tonsillar and other mucosal lymphoid tissues, local mucosal immune responses may also contribute to rapid viral clearance at the site of entry [26,27]. Further studies focusing on memory T-cell populations and mucosal immunity would help to clarify the mechanisms underlying the broad cross-protective capacity of the GPE[−] live vaccine.

Taken together, our data demonstrate that the currently used GPE[−] live vaccines provide robust protection against the circulating genotype 2.1d CSFV strains in Japan. Continuous monitoring of the antigenic characteristics of field strains remains essential, particularly in wild boar populations, where sustained viral transmission may facilitate viral evolution. Nevertheless, the present findings indicate that GPE[−] vaccination continues to play a key role in preventing clinical diseases and limiting viral spread in domestic pigs.

5. Conclusions

In this study, we demonstrated that the currently used GPE[−] live attenuated CSFV vaccines provide effective protection against a recently circulating genotype 2.1d strain in Japan that exhibits markedly reduced neutralizing reactivity to vaccine-induced antisera. Despite more than 32-fold lower in vitro neutralization titers compared with the homologous vaccine strain, vaccinated pigs showed complete clinical protection following challenge, with no detectable viremia or infectious virus shedding. These findings indicate that antigenic divergence alone does not necessarily compromise the protective efficacy of the GPE[−] vaccine. Protection was associated with the induction of robust immune responses prior to challenge and a rapid increase in neutralizing antibodies after infection, supporting the capacity of the GPE[−] vaccine to confer cross-protective immunity against antigenically distinct field strains.

Ongoing surveillance of circulating CSFV strains and periodic evaluation of vaccine efficacy remain essential, particularly in regions where sustained transmission in wild boar populations may promote viral evolution. Overall, the present study provides evidence that the GPE[−] live vaccine continues to play a critical role in preventing clinical disease and limiting viral spread in domestic pigs.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/vaccines14020170/s1>, Figure S1: Clinical signs of pig No. 15 (non-vaccinated control group); Figure S2: Pathological findings of pig No.15; Table S1: CSFVs used for neutralization testing with GPE[−] vaccine-induced antisera; Table S2: Time-course of detection of CSFV-specific genes from experimentally infected pigs; Table S3: Time-course of detection of swine GAPDH-specific genes from experimentally infected pigs; Table S4: Time-course of detection of CSFV-specific antibodies from experimentally infected pigs.

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Institutional Review Board Statement: All animal experiments were approved by the Animal Care and Use Committee of the NIAH prior to study initiation (authorization numbers: 20-036 and 24-027).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available within the article and its Supplementary Materials.

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Conflicts of Interest: This study was conducted in collaboration with Kyoritsu Seiyaku Corporation, and three co-authors (Emiko Ito, Miyabi Nishimura, and Yoshitaka Imaizumi) are employees of the company. The authors declare that they have no conflict of interest. The company does not influence the scientific interpretation or conclusions of this study.

Abbreviations

The following abbreviations are used in this manuscript:

CSF	classical swine fever
CSFV	classical swine fever virus
GPE [−]	guinea pig exaltation of Newcastle disease virus-negative strain
CPK	porcine kidney-derived cell line
DMEM/F-12	Dulbecco’s modified Eagle’s medium: nutrient mixture F12
ELISA	Enzyme-linked immunosorbent assay
MOI	multiplicity of infection
PBMC	Peripheral blood mononuclear cell
rRT-PCR	real-time reverse transcription-polymerase chain reaction
TCID ₅₀	50% tissue culture infectious dose
VNT	virus neutralization titer

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