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Pathogenicity and antigenic characterization of a novel highly virulent lineage 3 porcine reproductive and respiratory syndrome virus 2

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ABSTRACT

Background/purpose: Porcine reproductive and respiratory syndrome virus (PRRSV) is a pathogen with a negative economic impact on the global swine industry. In 2019, a suspected highly pathogenic strain, NPUST-108-929/2019 (108–929), was isolated from a pig farm in Pingtung with an outbreak of high mortality and analyzed. The characteristics of PRRSV 108–929 have barely been studied.

Methods: This study was to evaluate pathogenicity through animal challenge experiments using PRRSV 108–929 and antigenic characterization of this novel PRRSV.

Results: This PRRSV strain is PRRSV 2, belonging to lineage 3 based on open reading frame 5 sequence analysis. Four putative N-linked glycosylation sites (N32, N35, N44 and N51) are located on glycoprotein 5. Experimental results revealed that high fever occurred at 3 days postinoculation (dpi) in the high-titer inoculation (HIN) group (2×10^4 $TCID_{50}$/mL), 8 dpi in the high-titer contact (HC) group, 4 dpi in the low-titer inoculation (LIN) group (2×10^3 $TCID_{50}$/mL) and 9 dpi in the low-titer contact (LC) group. All pigs in each PRRSV 108–929 challenge and contact group showed severe clinical signs, such as high fever (>40.5 °C) and significant weight loss. Deaths occurred only in the HIN group; the survival rate was 60 %. All the piglets except the control group piglets showed high viremia titers (6.04–8.28 log^{10} copies/μL).

Conclusion: The pathogenic characteristics of PRRSV 108–929 suggest that it is a highly virulent PRRSV strain at both the farm and laboratory levels.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is one of the main illnesses in the modern swine industry worldwide and causes reproductive failure and respiratory distress, leading to severe economic loss. The causative pathogen, porcine reproductive and respiratory syndrome virus (PRRSV), is a positive sense, single stranded, enveloped RNA virus with an approximately 15 kb full genome length.[1] PRRSV has been classified into the *Betaarterivirus* genus, *Arteriviridae* family. PRRSVs are divided into 2 species, *Betaarterivirus suid* 1 (PRRSV 1, formerly called the European type), whose prototype is Lelystad, and *Betaarterivirus suid* 2 (PRRSV 2, formerly called the North American type), whose prototype is VR-2332.[2] The genomes of both species have only approximately 60 % homology, while *Betaarterivirus suid* 2 is the most frequently observed species in Taiwan.[3]

Open reading frame 5 (ORF5) encodes the major envelope glycoprotein 5 (GP5), which interacts with the host receptor to gain entry into the host cell.[4] GP5 is also a major inducer of neutralizing antibodies.[5] Three to five putative N-linked glycosylation sites (*N*-glycans) are located on the GP5 ectodomain, comprising the first forty residues of the mature protein.[6,7] In general, *N*-linked glycans are important for the folding, targeting and biological activity of proteins.[8] Hypoglycosylated

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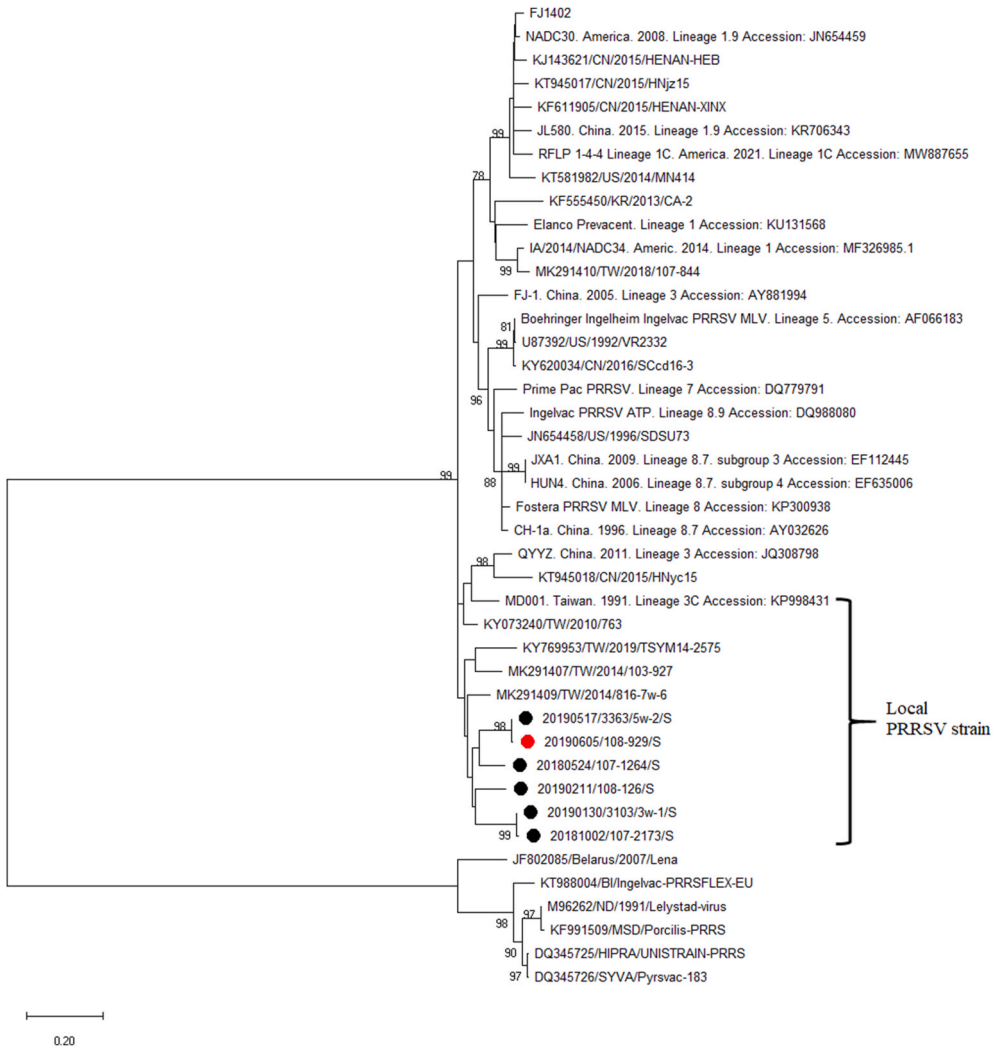


Fig. 1. A phylogenetic tree based on the ORF5 gene of PRRSV 2 sequences was constructed by maximum likelihood estimation (MLE) using Kimura 2-parameter (K2P) mode under the gamma distribution with 1000 bootstrapping replicates. The red circle represents the PRRSV 108–929 strain isolated in 2019 within the local PRRSV cluster. Black and red circle represents samples from the same farm. The scale bar indicates nucleotide substitutions per site.

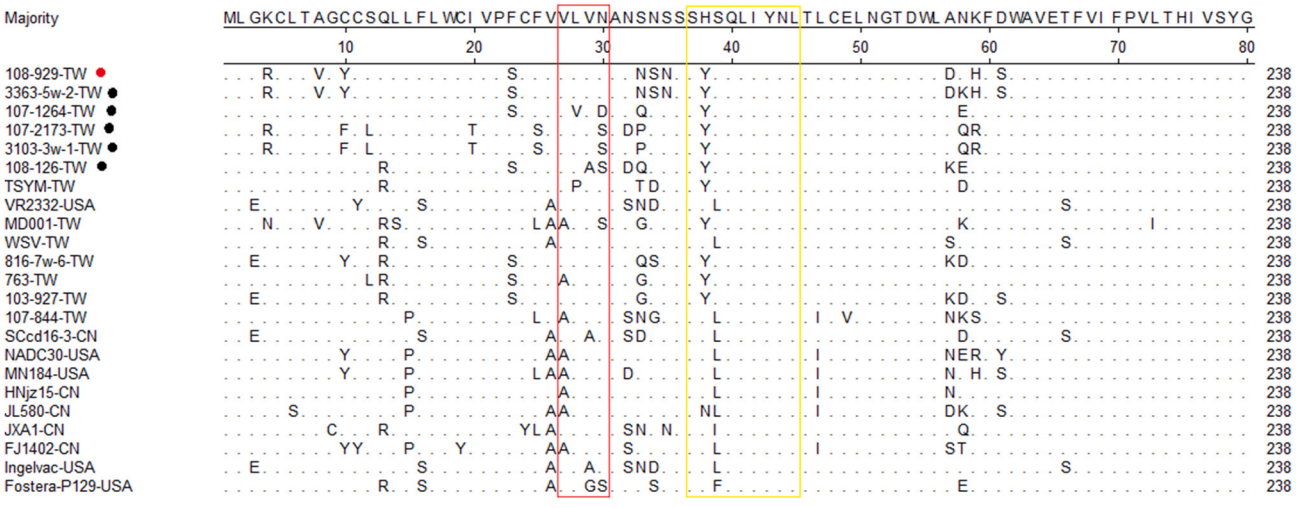
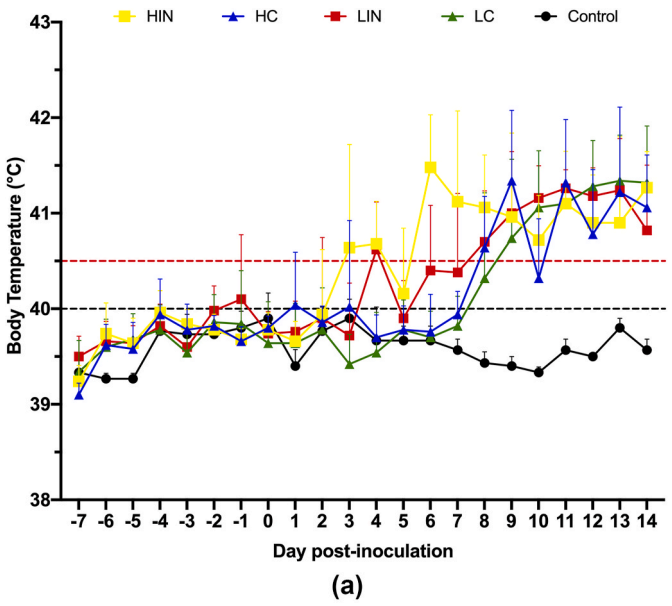


Fig. 2. Alignment of the partial amino acid sequences of the glycoprotein 5 of PRRSVs. The decoy epitope (DCE) is located at residues 27–30 (red box) and the primary neutralizing epitope (PNE) is located at residues 37–45 (yellow box). Dashes indicate identical bases. The red circle represents the PRRSV 108–929 strain isolated in this study. Black and red circle represents samples from the same farm.



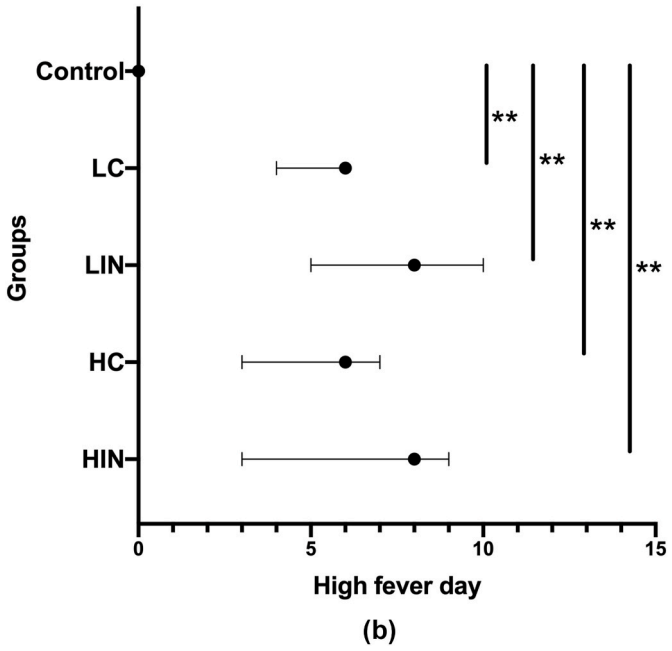


Fig. 3. Body temperature in pigs following the PRRSV 108–929 challenge (a). The threshold for fever was set at 40.0 °C (black dotted). The red dashed lines (high fever) represent body temperatures greater than 40.5 °C. High fever days in different groups of pigs following the PRRSV 108–929 challenge (b). Each error bar and black solid represent the range and median of the high fever days in each group, respectively. Statistically highly significant differences are marked as ** ($P < 0.01$).

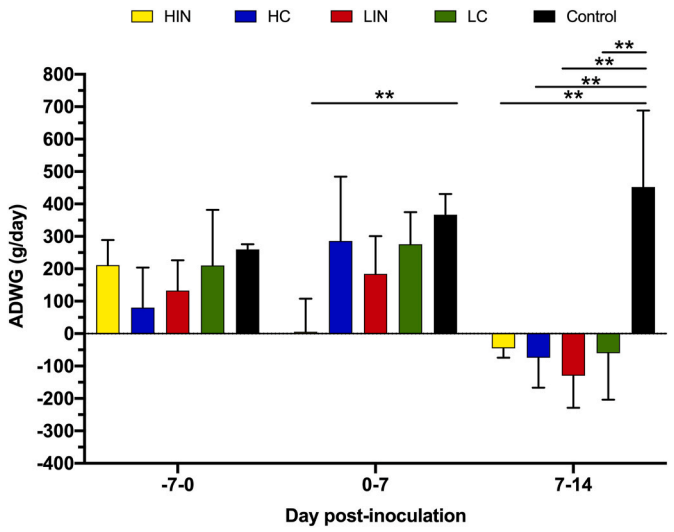


Fig. 4. The average daily weight gain in pigs following the PRRSV 108–929 challenge. Statistically highly significant differences are marked as ** ($P < 0.01$).

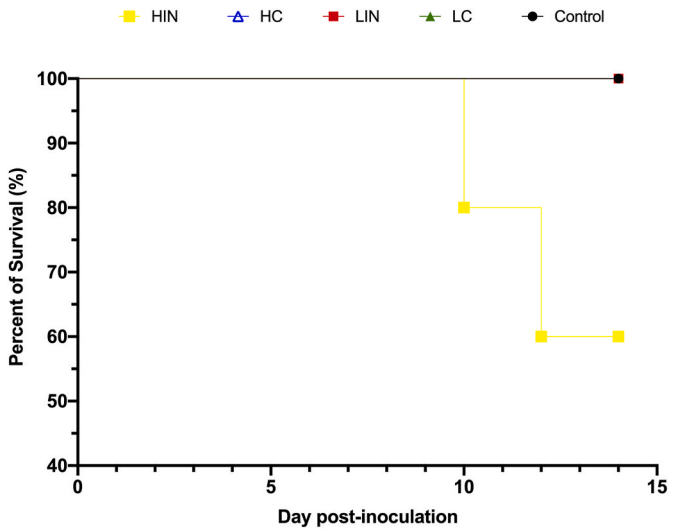


Fig. 5. Survival rates of pigs inoculated with PRRSV 108–929.

forms of the GP5 of PRRSVs are extremely sensitive to neutralization antibodies. Hypoglycosylated PRRSV (N34A and N51A) not only induces significantly higher levels of neutralizing antibodies against homologous mutant viruses and wild-type PRRSV but also increases the accessibility of the neutralizing epitope to convalescent antisera.[6] *N*-linked glycan moieties of the GP5 of PRRSV are important for the virus to escape the effect of neutralization antibodies.[6,7] In addition, the *N*-linked glycosylation site at residues 44 is critical for the recovery of viral infectivity.[6] Therefore, the *N*-linked glycans of the GP5 ectodomain play an important role in the proper functioning of the protein.

The clinical severity of PRRS can be influenced by several factors, including herd management, viral virulence, host immunity, the environment and secondary infection.[9] Several clinical signs can be seen in infected sows, such as agalactia, fever, abortion, farrow mummified fetuses and weak piglets.[10–12] Respiratory distress is mostly observed in nursery and growing pigs. Anorexia, lethargy, coughing and emaciation are common clinical signs, while highly pathogenic PRRSVs can also cause nervous system symptoms that are not commonly seen in infected swine.[9,13] In the summer of 2006, a highly pathogenic PRRSV (HP-PRRSV) was reported and emerged in the Chinese swine industry within one year. HP-PRRS causes high morbidity and mortality, which are accompanied by high fever and severe respiratory distress.[14,15] Nonsuppurative encephalitis is observed in animals experimentally inoculated with HP-PRRSV.[16] Currently, HP-PRRS has been reported in several Asian countries, such as Vietnam, Laos, Thailand and others.[17–19] The NADC-30 strain of PRRSV was first reported in the US in Iowa in 2008 and in China in 2013, causing abortion storms and severe respiratory distress.[20,21] Several recombinant viruses between HP-PRRSV and NADC-30 like strain of PRRSV have been reported, and its pathogenicity similar to that of HP-PRRSV has been observed.[22,23]

In Taiwan, the first PRRS outbreak occurred in 1991, and PRRS has

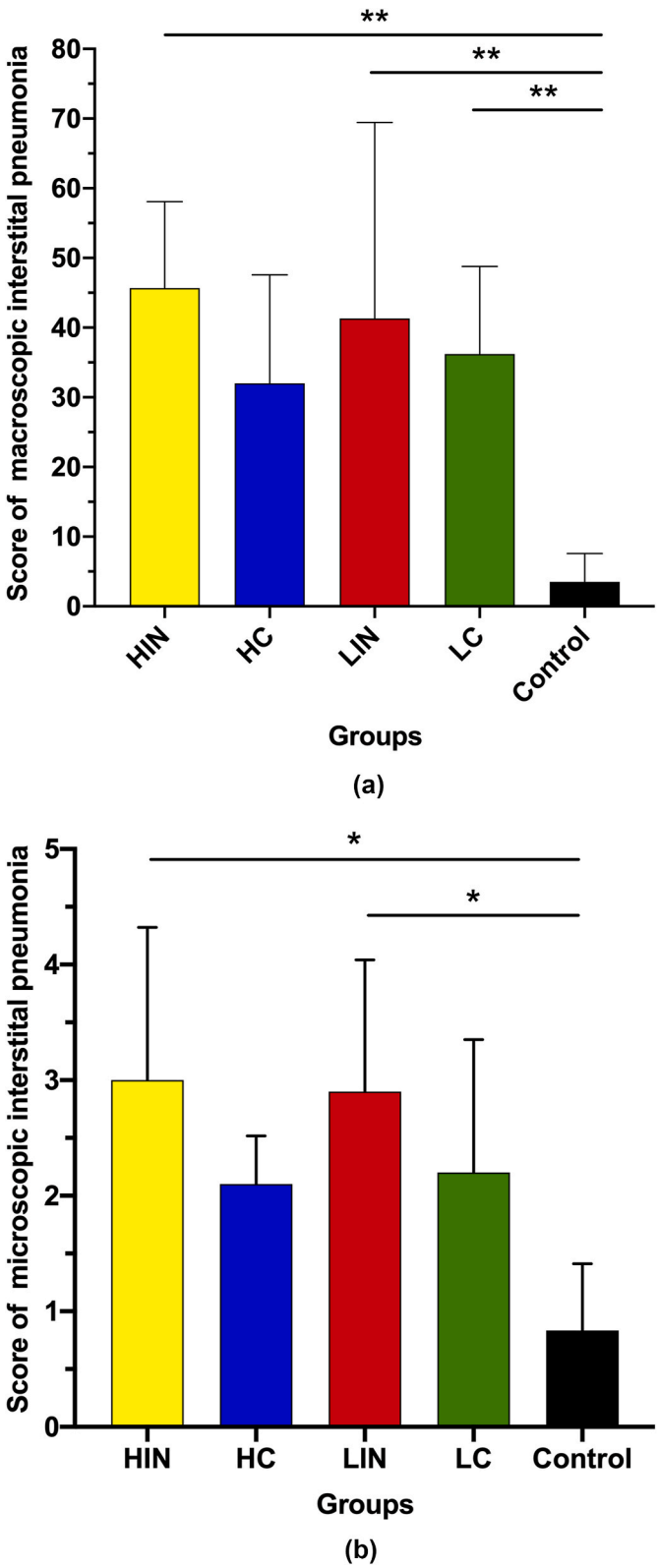


Fig. 6. Macroscopic (a) and microscopic (b) interstitial pneumonia scoring of each group after PRRSV 108–929 inoculation. Data are presented as the mean ± standard deviation (error bars). Statistically significant and highly significant differences are marked as * ($P < 0.05$) and ** ($P < 0.01$), respectively.

become a difficult issue in the Taiwanese swine industry.[24] The WSV and MD001 strains are prototypes of Taiwanese PRRSV, the WSV strain is genetically similar to the modified live vaccine strain used in Taiwan, and most of the wild PRRSVs in Taiwan in these two decades were most likely derived from MD001.[3] An NADC 30-like strain of PRRSV was also reported in Taiwan in 2018.[25] Although Taiwan has been considered free from HP-PRRSV, a comparably high-virulence strain, TSYM, was reported in 2019. The TSYM strain has been shown to induce high fever, high mortality, nonsuppurative encephalitis and high viremia titers similar to HP-PRRSV in animal challenge experiments.[26] In the present study, we determined the previously unidentified pathogen and investigated the pathogenicity of a recently isolated PRRSV 2 lineage 3 isolate.

2. Materials and methods

2.1. Sample collection

In June 2019, a 2300-sow farrow-to-finish farm had a serious PRRSV outbreak with high nursery pig mortality (37.1 %) and serious respiratory distress without significant neurological symptom. The pig herd routinely received PRRSV vaccine in sows with mass vaccination four times per year and one week old of piglets (Amervac-PRRS®, Hipra, Spain). Lung and serum samples that were submitted to the Animal Disease Diagnostic Center of National Pingtung University of Science and Technology (NPUST), and PRRSV was detected by real-time polymerase chain reaction as described previously.[27] Porcine circovirus type 2, *Mycoplasma hyopneumoniae* and *Mycoplasma suis* tested negative in this case.

2.2. ORF5 and non-structural protein 2 (nsp2) amplification and sequencing

The target sample was first amplified by PCR, and the ORF5 and nsp2 primers were described previously.[3,25] PCR products were then sent for sequencing after purification, ligation, transformation and blue and white screening. Sequences were then sent into MEGA 11 with the other reference PRRSV strains and aligned with Clustal W with the codon method.[28] After alignment, a phylogenetic tree was made by maximum likelihood method with the Kimura 2-parameter model and bootstrap values of 1000. The potential N-glycosylation site of GP5 was predicted by https://services.healthtech.dtu.dk/services/NetNGlyc-1.0/.

2.3. Virus isolation and purification

One milliliter of target serum sample was passed through 0.22 μm filter into a 25T flask containing 1×10^7 porcine alveolar macrophages (PAM) and cultivated in 10 % RPMI-1640 medium supplemented with 10 % fetal bovine serum (FBS) (Gibco Laboratories, Grand Island, USA) in a 5 % CO_2 atmosphere at 37 °C. PAM were obtained from the lungs of 6–8 week-old specific-pathogen-free pigs. The lung tissues were washed 3–5 times with PBS (pH 7.2). Each aliquot of lavage fluid was centrifuged for 10 min at 340×*g*. The resulting cells were mixed together and washed twice in PBS. Cells were resuspended in PRMI-1640 medium supplemented with 10 % FBS for viral propagation. When an 80 % cytopathic effect (CPE) was observed, the culture medium was frozen and thawed at −80 °C and 37 °C, respectively. After 3 cycles of freezing and thawing, the liquid was centrifuged, and the passage 1 virus was obtained as the supernatant. The methods were repeated until 3 passages were used to purify the virus.

2.4. Animal challenge experiment

Twenty-three PRRSV-free 4-weeks-old piglets were transferred into the negative pressure experimental animal house of the Animal Disease Diagnostic Center, National Pingtung University of Science and

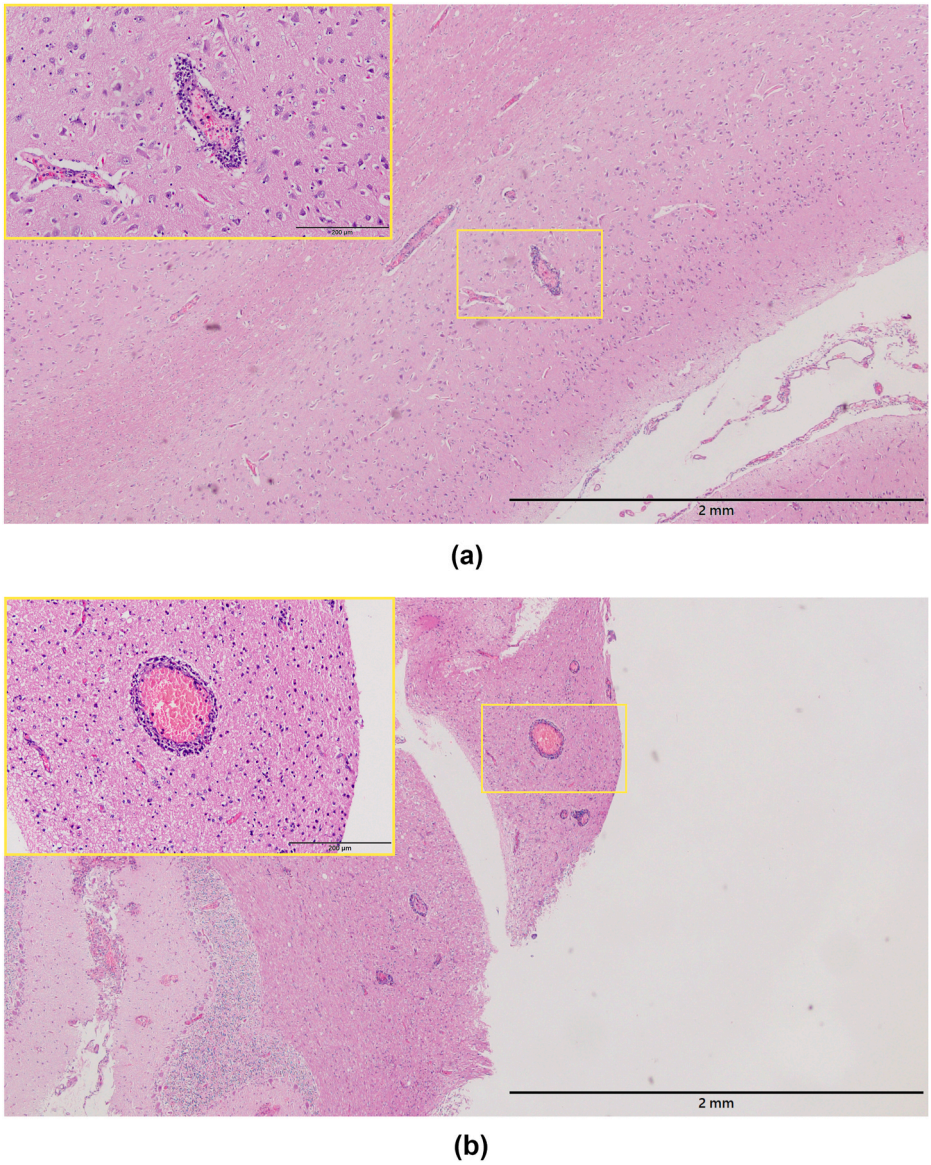


Fig. 7. Microscopic observation of the cerebrum (a) and cerebellum (b) of the dead pig from the HIN group, which showed severe perivascular cuffing. Inset: higher magnification of the perivascular cuffing (200 μm).

Technology at 7 days before inoculation (−7 days postinoculation, −7 dpi). Serum samples were collected from all the piglets for the confirmation of PRRSV antibody and viremia negativity. Piglets were divided into 5 groups: the control (C) (n = 3), low-titer intranasal inoculation (LIN) (n = 5), low-titer contact (LC) (n = 5), high-titer intranasal inoculation (HIN) (n = 5), and high-titer contact (HC) (n = 5) groups. Piglets in the LIN and HIN groups were inoculated with 2 ml of low titer (10^3 $TCID_{50}$/ml) or high titer (10^4 $TCID_{50}$/ml) PRRSV 108–929 strain, while piglets in the contact group stayed in the same pen with the corresponding inoculation group for contact infection. Serum samples were collected from all piglets at 0 dpi before inoculation and at 1, 3, 7, 10 and 14 dpi for the detection of PRRSV antibodies and to determine the viremia titer as described previously.[29] All piglets were weighted once a week for a comparison of the ADWG. Body temperature and clinical signs were measured and observed daily. Piglets were considered to have fever when their body temperature was above 40 °C and high fever when their body temperature was above 40.5 °C. All piglets were euthanized at 14 dpi.

2.5. Gross, microscopic examination and immunochemistry staining

During euthanasia, all piglets' lungs were scored for macroscopic lesions caused by viral interstitial pneumonia, which was characterized by multifocal to diffuse, tan to red mottled areas with irregular borders. All lung lobes were sectioned for pathological examination and interstitial pneumonia scoring. Gross and microscopic scoring systems were performed according to a previous study.[30] All the other organs were also sectioned for pathological examination. The tissue slices were deparaffinized and rehydrated by routine protocols and then rinsed with tris-buffered saline with 0.1 % Tween® 20 detergent (TBST). The antigen retrieval was performed by heating the slides in Trilogy™ (Cell Marque, Rocklin, CA, USA) at 95 °C for 5 min twice by a microwave (EZ-Retriever® System; BioGenex Laboratories Inc., San Ramon, CA, USA). After cooling to room temperature, the slides were washed with TBST three times and immersed with 3 % hydrogen peroxide (KYB, New Taipei City, Taiwan) for 15 min to remove the endogenous peroxidase activity in the tissue. Following TBST washing, the slides were blocked with 10 % normal serum (Dako) diluted in PBS at room temperature for 30 min to avoid non-specific binding. Antigen detection by primary

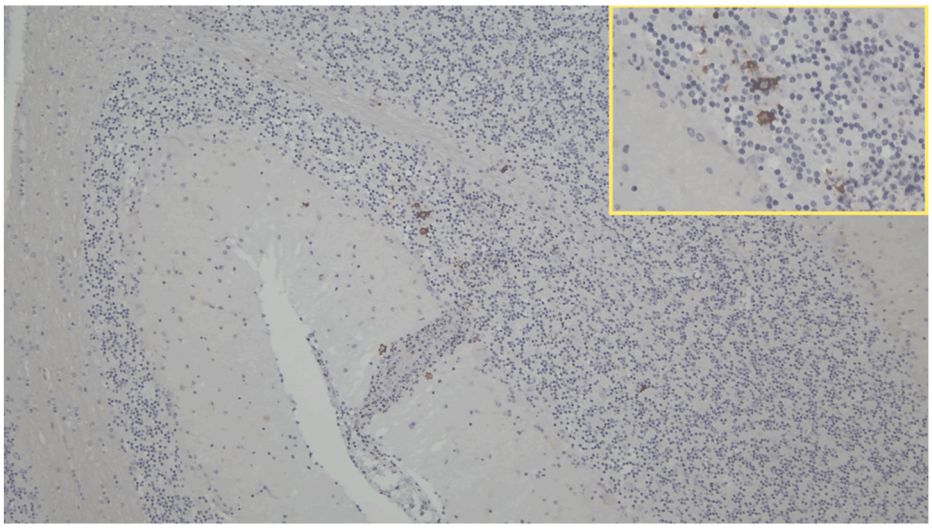


Fig. 8. PRRSV immunochemistry staining of the nervous tissues of the dead pig from the HIN group. Nervous tissue contained PRRSV antigen positive signals (brown) within the cytoplasm of macrophage-like cells in cerebellum.

antibodies was conducted by adding 800-fold diluted anti-PRRSV N protein antibody 1AC7 (Ingenasa, Madrid, Spain) at room temperature for 1-h incubation. Next, after washing with TBST three times, the tissue slides were developed by using EnVision® + Dual Link System-HRP (DAB+) (Dako), and then counterstained with hematoxylin for 20 s, followed by 5 min of washing with running tap water.

2.6. Detection of PRRSV antibodies and viremia titer measurement

Commercial enzyme-linked immunosorbent assay (ELISA) kit (IDEXX PRRS X3 Ab Test, IDEXX Laboratories, Inc., United States) were utilized for the measurement of PRRSV-specific antibody titers in all serum samples. All the operating procedures followed the commercial kit protocol. PRRSV-specific antibody titers were reported as sample-to-positive (S/P) ratios. An S/P ratio of 0.4 or higher was considered positive. Serum nucleic acids were extracted by a MagNA Pure 96 Instrument with a MagNA Pure 96 Total NA Isolation Kit (Roche Diagnostics GmbH, Mannheim, Germany). The PrimeScriptTM RT Reagent Kit (Takara Bio Inc., Shiga, Japan) was then utilized for the reverse transcription. All the serum samples were then quantified through real-time polymerase chain reaction.[27]

2.7. Statistical analysis

All the data collected in this study were analyzed by using GraphPad Prism 9.0 software. The average daily weight gain (ADWG), viremia titer and antibody titer were compared through two-way ANOVA. High fever days were compared using one-way ANOVA. The gross and interstitial pneumonia scores were analyzed through the Kruskal-Wallis test. P values < 0.05 and < 0.01 were considered statistically significant and highly significant, respectively.

3. Results

3.1. Virus isolation and genetic characterization

The target serum sample collected from this pig herd in June 2019, which had a PRRSV load of 2.09×10^9 copies/μL. This novel PRRSV was named PRRSV NPUST-108-929/2019 (108–929). This is a PRRSV 2 viral strain belonging to lineage 3 based on a completed ORF5 sequence analysis (data not shown). In comparison to the low nucleotide sequence similarity between PRRSV 108–929 and the reference foreign PRRSV, the homology levels between PRRSV 108–929 and the local PRRSV appeared to be much higher (Fig. 1). Surprisingly, PRRSV 108–929 was separated into different clusters when compared with the PRRSV isolated from the same farm (Fig. 1). It was shared only 89.1 % identity with its previous PRRSV isolate 108–126 (four month ago). The results of phylogenetic tree and alignment of the partial amino acid sequences between 108-126 and 108–929 were found unidentical (Figs. 1 and 2), so it was considered to be a newly invaded PRRSV strain. The decoy epitope (DCE) of PRRSV 108–929 was VLVN, which was similar to other local isolates (Fig. 2). The primary neutralizing epitope (PNE) was SYSQLIYNL, which was identical to previous isolates. The potential N-linked glycosylation site analysis showed that PRRSV 108–929 had four potential *N*-glycans at residues 32, 35, 44 and 51 (Fig. 2). This is the first study to identified N-glycans simultaneously at residues 32 and 35 of GP5 in a Taiwanese PRRSV strain (Fig. 2). Based on insertions and deletions in the nsp2 gene,[3] PRRSV 108–929 was type D pattern, which contained two deletions located between position 463–513 and between positions 530–549.

3.2. Clinical observation in the laboratory challenge model

Under laboratory challenge, high fever (more than 40.5 °C body temperature) was observed at 3 dpi for the first time in the HIN group (2×10^4 $TCID_{50}$/mL) (Fig. 3a). In the HIN group, the mean body temperature reached the threshold of high fever at 3 dpi for the first time, and high fever lasted until euthanasia except 5 dpi (Fig. 3a). In the LIN group (2×10^3 $TCID_{50}$/mL), the mean body temperature first reached the threshold of high fever at 4 dpi and then declined below the threshold. High fever was observed again at 8 dpi and then lasted until euthanasia (Fig. 3a). In the HC group, high fever was first observed at 8 dpi and then lasted until euthanasia except 10 dpi, while the LC group was first observed to have high fever at 9 dpi, which lasted until euthanasia (Fig. 3a). Fig. 3b summarizes the overall number of high fever days for each group. The overall numbers of high fever days for all groups were significantly higher than that of the control group. The median number of high fever days in the HC and LC groups were 6 days and 8 days for both the HIN and LIN groups (Fig. 3b). The HIN group showed a negative ADWG value at the first week after inoculation, which was significantly lower than that of the control and LC groups (Fig. 4). All the groups except the control group presented negative values at the second week after inoculation (Fig. 4). In the HIN group, two animals died at 10 and 12 dpi; accordingly, the survival rate was 60 % in this group, while no deaths occurred in the other groups (Fig. 5). Those 2 piglets were observed to have nervous system symptoms, paralysis and cyanosis

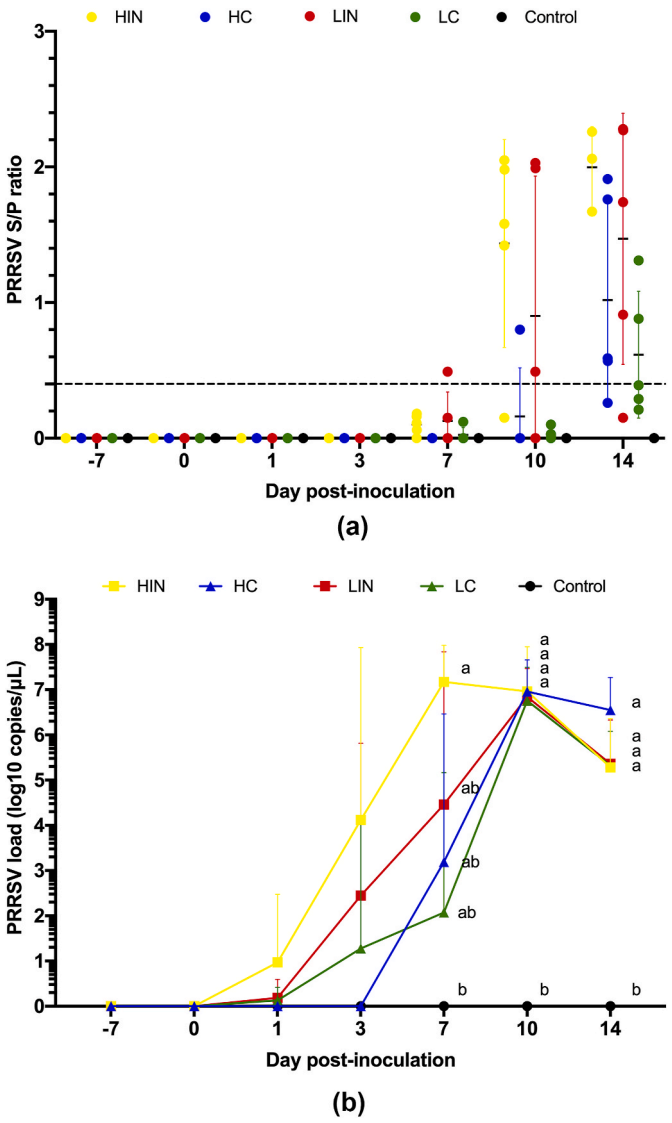


Fig. 9. PRRSV antibody level (a) in pigs following PRRSV 108–929 challenge. A sample-to-positive (S/P) ratio greater than 0.4 was interpreted as positive (dashed line). PRRSV load in the serum samples from each group in pigs following PRRSV 108–929 challenge (b). The error bars show the standard deviation. Different letters correspond to significant ($P < 0.05$) differences between groups for days postinoculation.

before death.

3.3. Gross and microscopic examination

Piglets in the challenge and contact groups showed obvious tan to red mottled areas with irregular borders lesions (data not shown). The macroscopic interstitial pneumonia score of the HIN, LIN and LC groups were significantly higher than that of the control group (Fig. 6a). The macroscopic interstitial pneumonia score indicated that moderate to severe interstitial pneumonia was present in the challenge and contact groups (Fig. 6a). All the microscopic interstitial pneumonia scores of the intranasal challenge groups were significantly higher than that of the control group, while there was also no significant difference in scores between the LIN and HIN groups (Fig. 6b). Perivascular cuffing with mononuclear cells infiltration in the cerebrum (Fig. 7a) and cerebellum (Fig. 7b) were observed in both of the dead piglets. The PRRSV immunochemistry staining results from neurological tissues showed the PRRSV antigen positive signals (brown) located within the cytoplasm of macrophage-like cells in cerebellum (Fig. 8). In addition, these two dead piglets had 1.2×10^6 copies/50 ng and 1×10^6 copies/50 ng of PRRSV from their brain homogenate tissues, respectively.

3.4. PRRSV antibody ELISA and PRRSV viremia

All the groups were negative for PRRSV-specific antibodies from −7 dpi to 3 dpi (Fig. 9a). At 7 dpi, the LIN group was the first group to present seroconversion, with a 20 % positive rate, while the other groups remained negative. At 10 dpi, seroconversion was observed in the HIN and HC group at 80 % and 20 %, respectively, while the LIN group presented a 60 % positive rate (Fig. 9a). At 14 dpi, the HIN group showed a 100 % positive rate for PRRSV-specific antibodies, and their antibody titer was significantly higher than those of the control and LC groups; the LIN group's positive rate remained 60 %, while that of the HC group increased to 60 %, and the LC group had the first seroconversion, 40 % (Fig. 9a). The control group piglets tested negative for PRRSV viremia throughout the whole experiment. The HIN group of pigs first showed low amounts of PRRSV viremia at 1 dpi (Fig. 9b). In the LIN and LC groups, PRRSV viremia was first observed at 3 dpi, with 20 % and 20 % positive rates, respectively, while PRRSV viremia was observed at 7 dpi in the HC group. All the challenge and contact groups reached a 100 % PRRSV viremia-positive rate at 7 dpi (Fig. 9b). The HIN group's viremia titer reached the peak value (7.17 ± 0.81 $\log^{10}$ copies/μL) at 7 dpi and was significantly higher than that of the control group, while the

Table 2
The potential N-linked glycosylation sites of the reference PRRSVs.

Strain	Accession No.	N-linked glycosylation sites
108–929	OM778882	32, 35, 44, 51
TSYM	KY769953	44, 51
MD001	KP998431	34, 44, 51
VR2332	EF536003	30, 33, 44, 51
WSV	KP998429	34, 44, 51
JXA-1	EF112445	30, 35, 44, 51
JL580	KR706343	34, 44, 51
FJ1402	KX169191	30, 34, 44, 51
JS2021NADC34	MZ820388	32, 33, 44, 51
NADC30	JN654459	34, 44, 51
NADC34	MF326985	32, 33, 44, 51, 57

Table 1
Review of the survival rate of reference PRRSVs under the challenge model.

Viral strain	Species	Inoculation dose	Inoculation route[a]	Survival rate	References
NPUST 108-929	PRRSV 2	2×10^4 $TCID_{50}$	IN	60 %	This study
JS2021NADC34	PRRSV 2	3×10^6 $TCID_{50}$	IN + IM	25 %	Yuan et al., 2022
TSYM	PRRSV 2	2×10^5 $TCID_{50}$	IN + IM	60 %	Hou et al., 2019
GZgy17	PRRSV 2	2×10^5 $TCID_{50}$	IN	80 %	Zhou et al., 2019
KU-N1202	PRRSV 2	$4 \times 10^{5.903}$ $TCID_{50}$	IN	100 %	Kwon et al., 2019
JL580	PRRSV 2	3×10^4 $TCID_{50}$	IN + IM	0 %	Zhao et al., 2015
Lena	PRRSV 1	1×10^6 $TCID_{50}$	IN	60 %	Karniychuk et al., 2010
NPUST 2789	PRRSV 1	2×10^5 $TCID_{50}$	IN	100 %	Hsueh et al., 2023

[a] IN: intranasal; IM: intramuscular.

other groups reached their peak (LIN, 6.84 ± 0.63 $\log^{10}$ copies/μL; HC, 6.96 ± 0.7 $\log^{10}$ copies/μL; LC, 6.75 ± 0.74 $\log^{10}$ copies/μL) at 10 dpi. Viremia titers declined gradually after they reached their peak value until euthanasia. All the challenge and contact groups' viremia titers were significantly higher than those of the control group at 10 and 14 dpi (Fig. 9b). The highest viremia titer induced by the 108–929 strain was 8.28 $\log^{10}$ copies/μL in the HIN group at 7 dpi, and only 2 piglets reached viremia titers above 8 $\log^{10}$ copies/μL, which were also the two piglets that died during the experiment.

4. Discussion

Since the first outbreak of PRRS in Taiwan, different strains of PRRSV have been found and reported continuously, but only one highly virulent PRRSV strain, TSYM, has been reported.[26] This study revealed that 108–929 could cause a lasting high fever, an extremely high viremia titer and nervous system symptoms. No exact definition of highly pathogenic or virulent PRRSV has been established, but it is usually defined by the performance of the strain in animal challenge experiments. Ruedas-Torres et al. reported criteria for high-virulence PRRSV, which included high mortality and morbidity at various ages on farms, hyperthermia, a drop in the ADWG in infected animals, severe interstitial pneumonia and lesions in other organs.[31] A serious outbreak occurred in the present pig herd in 2019, which caused a high nursery pig mortality rate of 37.1 % in June, which was suspected to be induced by this novel PRRSV. The main purpose of this investigation was to explain the pathogenicity of this novel PRRSV 2. Piglets challenged with 108–929 showed a prolonged high fever, and the data in this study revealed a high fever lasting for at least 6 days in the contact groups (Fig. 3b). The nervous system symptoms and lesions observed in both the cerebrum and cerebellum in the dead piglets illustrated that 108–929 may induce damage to the nervous system, causing nonsuppurative meningoencephalitis (Fig. 7a and b). A previous study indicated that HP-PRRSV had the ability to infect capillary endothelial cells and cross the blood-brain barrier.[16]

The clinical symptoms, viremia titer and mortality observed in this experiment were similar to data for the TSYM strain and HP-PRRSV, which demonstrated that 108–929 could be defined as a highly pathogenic PRRSV strain.[22,26,32] The HIN group in this study showed earlier viremia and high fever than the other groups, and the HIN group was the only group that showed mortality during the experiment. All of the contacts had been infected and later showed clinical signs. These data revealed that this experimental model may be a suitable model for PRRSV challenge experiments to show obvious differences between high and low doses or inoculation and contact pathways. Table 1 summarizes the challenge studies of the different PRRSVs. When comparing the survival rate (60 % versus 60 %), inoculation route (IN versus IN + IM) and inoculation dose (2×10^4 $TCID_{50}$ versus 2×10^5 $TCID_{50}$), the mortality of PRRSV 108–929 was higher than that of PRRSV TSYM. Taken together, these experiments in pigs showed that PRRSV 108–929 had higher virulence than the previous local PRRSV 2 TSYM strain.

The number of *N*-glycans of the GP5 of PRRSV might affect the proper functioning of the protein. Most PRRSVs contain three putative N-lined glycans at their GP5.[6] Previous studies have indicated that *N*-glycans located at upstream of residues 44 and 51 are important for the virus to escape antibody neutralization.[7] Interestingly, PRRSV 108–929 had four potential *N*-glycans at residues 32, 35, 44 and 51 (Fig. 2, Table 2). *N*-glycans at residues 44 and 51 are more conserved among PRRSVs.[33] The function of N44 is most critical for the recovery of viral infectivity.[6] The *N*-glycan at N51 is important for the virus to escape the effect of neutralization antibodies, and N51 mutant PRRSV induces significantly higher levels of neutralizing antibodies to homologous mutant virus.[6] Surprisingly, compared to a previous study,[3] *N*-glycans simultaneously observed at residues 32 and 35 were first identified in the Taiwanese PRRSV strain in the present study. Residues 32–35 of GP5 have been previously detected as a region evolving under strong evolutionary pressure,[34] and both residues 32 and 35 are located at positive selection sites. Table 2 summarizes the potential *N*-glycans from reference PRRSVs. *N*-glycans at residues 32 and 35 have also been found in the highly pathogenic PRRSV strains JS2021NADC34 and JXA-1, respectively,[33] but they do not simultaneously occur in the same PRRSV strain. JS2021NADC34 is a Chinese NADC34-like PRRSV isolated in Jiangsu Province in 2021.[35–37] JXA-1 was a prototypical highly pathogenic strain in China in 2006.[14] Similar to our isolate, PRRSV 108–929, PRRSV strains JS2021NADC34 and JXA-1 both caused lasting fever, reduced body weight, high morbidity, high mortality, severe interstitial pneumonia and high viremia in infected pigs.[14,37] Whether *N*-glycans at residues 32 and/or 35 are critical for viral virulence remains to be further investigated.

Many studies have shown that the recombination and mutation of some strains leads to a novel virulent strain that causes endemic outbreaks.[38,39] Where this novel virus came from remains unknown. However, PRRSV 108–929 shared only 89.1 % identity with previous isolates based on ORF5 sequence analysis. This might imply that a new PRRSV strain invaded this pig farm. Further study is warranted to determine the full-length genome characteristics to confirm whether any recombination occurred between PRRSV 108–929 and other PRRSVs. In this decade, Taiwan has been reported 2 high-virulence PRRSV strains, including 108–929, and this phenomenon might be an alert to the Taiwanese swine industry. As the PRRSV strains that emerged in Taiwan became complicated, the risk of recombination increased, and a novel PRRSV with uncertain pathogenicity appeared. The importation of instruments, breeding pigs or anything used on farms should be done more carefully, and sufficient quarantine time and disinfection could be an important issue for PRRSV infection status. In addition, the protective efficacy of current PRRSV vaccines against this novel virus remains to be investigated.

In Summary, in this pathogenicity study, 5-week-old pigs were intranasally inoculated with 2×10^4 $TCID_{50}$ of PRRSV 108–929, and the results showed that it caused hyperthermia, significantly reduced body weight, high viremia, high mortality, severe interstitial pneumonia and nonsuppurative meningoencephalitis. These results suggested that PRRSV 108–929 is a highly virulent PRRSV strain.

CRediT authorship contribution statement

Yon-Yip Chan: Data curation, Methodology, Writing – original draft. **Cheng-Yao Yang:** Data curation, Investigation, Methodology. **Chuen-Fu Lin:** Data curation, Investigation. **Sheng-Yuan Wang:** Methodology. **Wei-Hao Lin:** Methodology. **Ming-Tang Chiou:** Conceptualization, Investigation, Supervision, Validation, Writing – review & editing. **Chao-Nan Lin:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

Ethics approval

The animal experimental procedure was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the National Pingtung University of Science and Technology with approval No. NPUST-109-099.

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Declaration of competing interest

None declared.

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References

1. Benfield DA, Nelson E, Collins JE, et al. Characterization of swine infertility and respiratory syndrome (SIRS) virus (isolate ATCC VR-2332). *J Vet Diagn Invest*. 1992; 4:127–133.
2. Adams MJ, Lefkowitz EJ, King AMQ, et al. Changes to taxonomy and the international code of virus classification and nomenclature ratified by the international committee on taxonomy of viruses. *Arch Virol*. 2017;162:2505–2538.
3. Deng MC, Tsai HJ, Chang C, et al. Molecular epidemiology of porcine reproductive and respiratory syndrome viruses isolated from 1991 to 2013 in Taiwan. *Arch Virol*. 2015;160:2709–2718.
4. Dea S, Gagnon CA, Mardassi H, Pirzadeh B, Rogan D. Current knowledge on the structural proteins of PRRSV comparison of the North American and European isolates. *Arch Virol*. 2000;145:659–688.
5. Lopez OJ, Ostrowski M, Galeota J a, Jar a M, Platt KB, Osorio F a. Identification of neutralizing and nonneutralizing epitopes in the porcine reproductive and respiratory syndrome virus GP5 ectodomain identification of neutralizing and nonneutralizing epitopes in the porcine reproductive and respiratory syndrome virus GP. *J Virol*. 2002;76:4241–4250.
6. Ansari IH, Kwon B, Osorio FA, Pattnaik AK. Influence of N-linked glycosylation of porcine reproductive and respiratory syndrome virus GP5 on virus infectivity, antigenicity, and ability to induce neutralizing antibodies. *J Virol*. 2006;80: 3994–4004.
7. Vu HLX, Kwon B, Yoon K-J, Laegreid WW, Pattnaik AK, Osorio FA. Immune evasion of porcine reproductive and respiratory syndrome virus through glycan shielding involves both glycoprotein 5 as well as glycoprotein 3. *J Virol*. 2011;85:5555–5564.
8. Braakman I, van Anken E. Folding of viral envelope glycoproteins in the endoplasmic reticulum. *Traffic*. 2000;1:533–539.
9. White MEC. The clinical signs and symptoms of blue-eared pig disease (PRRS). *Pig Vet J*. 1992;28:62–68.
10. White MEC. PRRS-clinical update. *Pig Vet J*. 1992;29:179–187.
11. Keffaber K. Reproduction failure of unknown etiology. *Am Assoc Swine Pr Newsl*. 1989;1:1–9.
12. Hopper SA, White ME, Twiddy N. An outbreak of blue-eared pig disease (porcine reproductive and respiratory syndrome) in four pig herds in Great Britain. *Vet Rec*. 1992;131:140–144.
13. Thanawongnuwech R, Halbur P, Andrews J. Immunohistochemical detection of porcine reproductive and respiratory syndrome virus antigen in neurovascular lesions. *J Vet Diagn Invest*. 1997;9:334–337.
14. Tian K, Yu X, Zhao T, et al. Emergence of fatal PRRSV variants: unparalleled outbreaks of atypical PRRS in China and molecular dissection of the unique hallmark. *PLoS One*. 2007;2:e526.
15. Yang HC. Prevalence status of “swine high fever syndrome” (atypical PRRS). *Swine IndSci*. 2007;1:77–80.
16. Cao J, Li B, Fang L, Chen H, Xiao S. Pathogenesis of nonsuppurative encephalitis caused by highly pathogenic Porcine reproductive and respiratory syndrome virus. *J Vet Diagn Invest*. 2012;24:767–771.
17. Metwally S, Mohamed F, Faaberg K, et al. Pathogenicity and molecular characterization of emerging porcine reproductive and respiratory syndrome virus in Vietnam in 2007. *Transbound Emerg Dis*. 2010;57:315–329.
18. Ni J, Yang S, Bounlom D, et al. Emergence and pathogenicity of highly pathogenic porcine reproductive and respiratory syndrome virus in Vientiane, Lao People’s Democratic Republic. *J Vet Diagn Invest*. 2012;24:349–354.
19. Nilubol D, Tripipat T, Hoonsuwan T, Kortheerakul K. Porcine reproductive and respiratory syndrome virus, Thailand, 2010-2011. *Emerg Infect Dis*. 2012;18: 2039–2043.
20. Brockmeier SL, Loving CL, Vorwald AC, et al. Genomic sequence and virulence comparison of four Type 2 porcine reproductive and respiratory syndrome virus strains. *Virus Res*. 2012;169:212–221.
21. Zhou L, Wang Z, Ding Y, Ge X, Guo X, Yang H. NADC30-like strain of porcine reproductive and respiratory syndrome virus, China. *Emerg Infect Dis*. 2015;21:2256.
22. Sun Z, Wang J, Bai X, et al. Pathogenicity comparison between highly pathogenic and NADC30-like porcine reproductive and respiratory syndrome virus. *Arch Virol*. 2016;161:2257–2261.
23. Wang H, Liu Y, Tang Y, et al. A natural recombinant PRRSV between HP-PRRSV JXA1-like and NADC30-like strains. *Transbound Emerg Dis*. 2018;65:1078–1086.
24. Chang CC, Chung WB, Lin MW, et al. Porcine reproductive and respiratory syndrome (PRRS) in Taiwan. I. Viral isolation. *J Chin Soc Vet Sci*. 1993;19:268–276.
25. Lin WH, Shih HC, Wang SY, et al. Emergence of a virulent porcine reproductive and respiratory syndrome virus in Taiwan in 2018. *Transbound Emerg Dis*. 2019;66: 1138–1141.
26. Hou FH, Chia MY, Lee YH, Liao JW, Lee WC. A comparably high virulence strain of porcine reproductive and respiratory syndrome virus isolated in Taiwan. *Comp Immunol Microbiol Infect Dis*. 2019;65:96–102.
27. Lin CN, Lin WH, Hung LN, Wang SY, Chiou MT. Comparison of viremia of type II porcine reproductive and respiratory syndrome virus in naturally infected pigs by zip nucleic acid probe-based real-time PCR. *BMC Vet Res*. 2013;9:1.
28. Tamura K, Stecher G, Kumar S. MEGA11: molecular evolutionary genetics analysis version 11. *Mol Biol Evol*. 2021;38:3022–3027.
29. Hsueh FC, Kuo KL, Hsu FY, et al. Molecular characteristics and pathogenicity of porcine reproductive and respiratory syndrome virus (PRRSV) 1 in taiwan during 2019–2020. *Life*. 2023;13:1–11.
30. Halbur PG, Paul PS, Frey ML, et al. Comparison of the antigen distribution of two US porcine reproductive and respiratory syndrome virus isolates with that of the lelystad virus. *Vet Pathol*. 1996;33:159–170.
31. Ruedas-Torres I, Rodríguez-Gómez IM, Sánchez-Carvajal JM, et al. The jigsaw of PRRSV virulence. *Vet Microbiol*. 2021;260, 109168.
32. Brockmeier SL, Loving CL, Palmer MV, et al. Comparison of Asian porcine high fever disease isolates of porcine reproductive and respiratory syndrome virus to United States isolates for their ability to cause disease and secondary bacterial infection in swine. *Vet Microbiol*. 2017;203:6–17.
33. Zhou L, Yang Y, Xia Q, et al. Genetic characterization of porcine reproductive and respiratory syndrome virus from Eastern China during 2017-2022. *Front Microbiol*. 2022;13, 971817.
34. Mu C, Lu X, Duan E, et al. Molecular evolution of porcine reproductive and respiratory syndrome virus isolates from central China. *Res Vet Sci*. 2013;95: 908–912.
35. Wang FX, Qin LT, Liu Y, et al. Novel Nsp2 deletion based on molecular epidemiology and evolution of porcine reproductive and respiratory syndrome virus in Shandong Province from 2013 to 2014. *Infect Genet Evol*. 2015;33:219–226.
36. Zhu Z, Yuan L, Hu D, et al. Isolation and genomic characterization of a Chinese NADC34-like PRRSV isolated from Jiangsu province. *Transbound Emerg Dis*. 2022; 69:e1015–e1027.
37. Yuan L, Zhu Z, Fan J, et al. High pathogenicity of a Chinese NADC34-like PRRSV on pigs. *Microbiol Spectr*. 2022;10, e0154122.
38. Zhao K, Ye C, Chang X-B, et al. Importation and recombination are responsible for the latest emergence of highly pathogenic porcine reproductive and respiratory syndrome virus in China. *J Virol*. 2015;89:10712–10716.
39. Dong J, Wang Y, Yu L, et al. Pathogenicity of a newly emerged recombined porcine reproductive and respiratory syndrome virus strain (subgenotype III) in China. *Vet Microbiol*. 2017;210:162–166.