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A comparably high virulence strain of porcine reproductive and respiratory syndrome virus isolated in Taiwan

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) has been endemic in Taiwan since 1991. This study aimed to present a highly virulent PRRSV in Taiwan based on farm data collection and both *in vitro* and *in vivo* evaluations in virus challenge studies. This virulent PRRSV strain was first noticed on Farm TSYM due to continuously high nursery mortality rate and severe PRRSV-associated pneumonia. In phylogenetic surveillance, the PRRSV TSYM-strain remained in the predominant position for years, even with several other PRRSV strain invasions. In laboratory challenge trials, the TSYM-strain led to prolonged pyrexia, growth retardation, high mortality rates and high viremia titer that similar to the highly pathogenic PRRSV. The TSYM-strain isolate also triggered early interleukin-10 up-regulation and significantly higher infection rates under *in vitro* experiments. This study provides information of a comparably virulent strain in Taiwan and its appearance in both farm and laboratory levels.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is one of the most important diseases in the modern swine industry. The causative pathogen, PRRS virus (PRRSV), is a small, enveloped, single-stranded, positive-sense RNA virus, which has been classified as one member of the *Arterividae* family, *Nidovirales* order [1,2]. Infection with PRRSV predominantly leads to respiratory disorders in pigs of all ages and reproductive loss in breeding populations [2,3]. The severity of PRRS is largely influenced by multiple factors including viral virulence, host genetic variation, age and immune status, management and environmental conditions [4,5]. PRRSV has also been broadly proven to serve as a primary pathogen and impair the respiratory system, which interacts with other infectious agents to develop more complicated pathogenicity [2].

PRRSV is comprised of two major genotypes: genotype I (European type) and genotype II (North American type). PRRSV is characterized by remarkable evolution rates that cause great diversity in antigenicity and pathogenicity and confers variable immunological cross-reactions among different isolates [6]. This "quasispecies" situation of PRRSV results in difficulties for disease control and vaccine development.

The ability of PRRSV to transmit and to cause disease is greatly variable among strains. Most endemic PRRSV strains trigger impairment of nursery pigs and circulate within neighboring areas or certain production systems due to the presence of partial protection in endemic areas [7]. However, there are several important events of epidemic PRRS in the history of PRRSV epidemiology which have caused devastating diseases and pandemic outbreaks by specific PRRSV strains. For instance, there was the "high fever disease" in Asia caused by highly pathogenic PRRSV (HP-PRRSV). The representative clinical signs of HP-PRRS included high fever (40–42 °C), rubefaction, severe depression, respiratory disorders and high mortality (20–100%). It even caused deaths in grown pigs, which differs from typical PRRSV infection [8,9]. The aberrant ability to trigger higher viral loads and greater pathogenic changes has led to HP-PRRSV being generally known as a virulent strain [10].

In Taiwan, PRRSV has been circulating in most farms since the first identification by Chang et al. in 1991 (Taiwan Veterinary Journal). Besides the prototypic strains MD001 and WSV, many PRRSV strains in Taiwan have also been studied in molecular epidemiological analysis [11]. However, the virulence of Taiwanese isolates remains barely mentioned so far. This study aimed to present a comparably high virulence PRRSV in Taiwan based on farm data collection and both *in vitro* and *in vivo* evaluations in virus challenge studies.

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2. Materials and methods

2.1. Farms

The field observations were conducted on Farm TSYM, a farrow-to-finish farm with an inventory of 15,000 pigs, in Taiwan. This farm is located within an isolated area and distant from other pig farms. Batches of piglets weaned at the age of 4 weeks were transferred into a continuous feeding nursery house. Since 2013, the farm had been suffering from severe respiratory disorders and continuously high mortalities in the nursery phase. For further observation, a total of 561 weaned pigs were monitored daily until they were moved to the fattening unit at the age of 11 weeks. In addition, Farm TSYM routinely received batches of 8–10 week-old pig herds from at least four PRRS-endemic farms. Afterwards, pigs were periodically sampled for PRRSV phylogenetic surveillance.

2.2. Phylogenetic analysis

The PRRSV ORF5 gene was detected through RT-PCR techniques and then submitted for gene sequencing. Sequence alignment was performed with the MUSCLE program and analyzed with the Maximum-likelihood method and bootstrapped with 1000 replicates *via* MEGA 5.2 software. All sequences were compared with representative strains of different lineages published on GenBank, National Center for Biotechology Information, and classified based on previously described methods [12].

2.3. Virus isolation and animal challenge study

The PRRSV TSYM were isolated and stocked after four passages on porcine alveolar macrophages (PAM). In addition, a contrast PRRSV isolate, HBZF-strain, was also isolated from a PRRS-endemic farrow-to-finish pig farm in Taiwan in 2014, which reared approximately 11,000 pigs. The serological profiles indicated that all pigs were naturally infected with PRRSV during the nursery phase. However, no obvious PRRS-associated clinical signs were complained about on this farm, which was without PRRSV vaccine implementation, and experienced only minimal losses (less than 5%) in the nursery phase over several years. The HBZF isolate served as a comparably low virulence Taiwanese strain in this study. Both viral inocula of TSYM and HBZF were confirmed as negative for classical swine fever virus, pseudorabies virus, type 2 porcine circovirus and swine influenza virus by PCR or RT-PCR assays.

All data in the challenge studies were collected from two independent trials. In trial I, eight male early-weaned conventional piglets were transferred into the experimental animal house at three weeks of age. Serum samples of all pigs were collected for confirmation of PRRSV antibody and for being nucleic acid free. Pigs were divided into three groups, mock (n = 2), HBZF (n = 3) and TSYM (n = 3) groups, and fed in separate rooms. Two PRRSV inocula were administered (IN + IM) with 2×10^5 50% tissue culture infectious dose at five week-old. In trial II, ten specific-pathogen-free pigs were divided into mock (n = 5) and TSYM groups (n = 5), and challenged at eight week-old. Both mock groups were administered the same volume of filtered PAM freeze-thaw lysates. Serum samples of all pigs were collected at 0, 7, 14 and 21 day-post-challenge (DPC) for quantification of PRRSV viremia. Pathological examination was performed at 21 DPC. Lung tissues were collected for histopathological examination and lesion scoring. Both trials were conducted within the experimental house of the Graduate Institute of Veterinary Pathobiology.

The experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of National Chung Hsing University (IACUC number: 103 = 98 & 105-139).

2.4. Clinical measurements

Body temperatures and clinical signs were recorded daily by the same investigators, who were blind to the experiment groups. The baseline temperatures were measured individually days before challenge. Rises of body temperature were defined as moderate (0.5–1 ℃) and high (> 1 ℃) and applied to the calculation of fever index by using the following formula: [(Days of moderate fever) × 1 + (Days of high fever) × 2] / (Living days). The body weights were measured at 0 and 21 DPC for the average daily weight gain calculation.

2.5. Euthanasia and pathological examination

Pigs showing a prolonged inability to move, hypothermia, severe weight loss or any indications of intense pain were humanely euthanized by electrical stunning and exsanguination in this study. For field observations, all pigs that died or were culled were necropsied and scored for areas of bronchopneumonia lesions and severity of interstitial pneumonia by four pathologists following the previous study with modifications. The macroscopic score of interstitial pneumonia ranged from 0 to 4 (0, normal; 1, mild and local; 2, slightly meaty texture and extensive; 3, profound firm texture and diffuse). The macro- and microscopic lung lesion scoring method used in the animal challenge model was described in a previous study [13].

2.6. Quantification of PRRSV viremia

Viral RNA was extracted from serum samples by using QIAamp Viral RNA Mini Kit (QIAGEN) and reverse transcribed with the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. A 20 μL PCR reaction mixture containing 1 × SYBR green master mix (Kapa), 250 nM of each primer (Forward: 5′- AGCCAGTCAATCAGCTG TGCC-3′ / Reverse: 5′-AGGCGGTATGGATTGACGAC-3′) and 5 μL of cDNA template were set up. The PCR was performed with LightCycler 480 (Roche) through a 40-cycle amplification program and followed by a melting curve for verifying specificity. Serial 10-fold dilution of the OD-titrated plasmid constructed with ORF7 of Taiwanese PRRSV was used as the standard on each plate.

2.7. In vitro study on cell viability, viral replication and cytokine mRNA profiles of PAM

Thawed PAM were inoculated on Teflon plates with 0.1 multiplicity of infection of either HBZF or TSYM viral inoculum in triplication. Inoculated cells were harvested and stained with propidium iodide (1 μg/mL) and SDOW-17 monoclonal antibody (RTI) for cell viability and PRRSV titration assay, respectively. The FSC/SSC-gated PAM population were acquired in FACScan flow cytometry (Becton Dickinson) for at least 10,000 events. The portions of PI negative and SDOW17-FITC positive populations were measured for quantification of live cells and PRRSV infected cells, respectively.

Method of cytokine mRNA relative quantification was referenced from a previous study [14]. An internal house-keeping gene, glyceraldehyde 3-phosphate dehydrogenase, and mock control were simultaneously set in the same plate with target sample genes and calculated by using $2^{-\Delta\Delta Ct}$ method.

2.8. Statistical analysis

Data collected in this study was all analyzed using IBM SPSS statistical software (version 20) for statistical analysis. Student's t tests were performed for continuous data by comparison between groups. *P* values less than 0.05 were considered to be statistically significant.

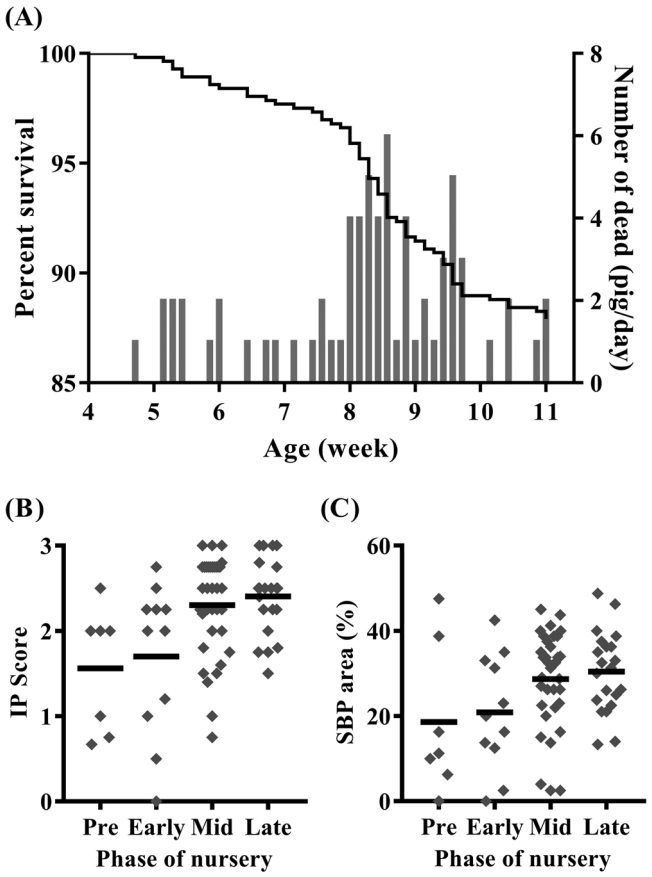

Fig. 1. In-field observation of PRRSV TSYM-strain endemic farm. Mortality records of a total of 561 weaned pigs are summarized. The line indicates the accumulative survival rates and the grey bars reveal the numbers of pigs that died or were culled on each day of age (A). The pigs that died (N = 54) or were culled (N = 13) were divided into pre-weaning (≤4 wk) and early (5–6 wk), mid (7–8 wk), late (> 9 wk) phases of nursing and scored for macroscopic interstitial pneumonia (B) and suppurative bronchopneumonia (C). The individual values and average scores are indicated as grey dots and horizontal lines, respectively.

3. Results

3.1. In-field observation

The average mortalities of Farm TSYM were approximately 31.3 ± 18.5% in the nursery since 2014 and could reach up to 82% in some batches under harsh environmental conditions. The average mortalities in the growing/finishing phases were 2.7 ± 1.4 and 0.9 ± 0.4%, which contributed to limited loss on this farm (data not shown). In the record of nursery mortalities, most weaned pigs died or were culled at the age of seven to ten weeks (mid- or late nursery phases) (Fig. 1A). Pigs in this period showed more severe interstitial pneumonia complicated with bronchopneumonia and polyserositis compared to that of pre- or early weaning phases (Fig. 1B and C).

3.2. Phylogenetic surveillance on farm TSYM

A total of 30 ORF5 genes were detected and analyzed for phylogenecity during the period from 2013 to 2017 (Fig. 2). The results indicated 27 sequences formed a monophyletic group (TSYM-cluster) with an intra-cluster divergence of 2.51% (0.17–8.39%) in nucleotide distances. Phylogenetic analysis revealed TSYM-group belonged to lineage 3 of type II PRRSV and shared an 88% similarity with MD001, the prototypical strain in Taiwan [12]. TSYM isolates were also distinct from the published virulent PRRSV strains in neighboring countries, including HP-PRRSV, NADC30 and MN-184 strains. Among the submitted sequences, three outside strains were also detected in this study. The NSL- and DHF-strains were detected in samples that were collected in the first two days after pigs were introduced from other PRRSV-endemic farms. The TSYM-150254C sequence shared 99.3% of its identity with the modified live virus vaccine (MLV) strain, which may because of a temporary implementation of MLV vaccination in 2015.

3.3. Clinical observations in the laboratory challenge model

The body temperature remained steady with an average of 39 °C before challenge and showed rises after being challenged with either PRRSV strain within two days. Compared to the short-lasting and moderate fever in the HBZF group, the TSYM groups in both trials showed prolonged and severe pyrexia, which could reach up to 42 °C in some individuals (Fig. 3A). The fever index was calculated to minimize the biases of underestimation caused by pig death. Both TSYM groups showed higher fever indexes than that of HBZF (Fig. 3B). Pigs challenged with TSYM virus also revealed profound depression, anorexia and respiratory disorders for 7–10 days, and the severity of the respiratory status gradually deteriorated to abdominal breathing and even open-mouthed breathing before being euthanized (data not shown). Severe cachexia and wasting were noted in the TSYM-challenged pigs (Fig. 3C). With two pigs dead and one euthanized in trial I and one dead and one euthanized in trial II, the mortality rates of the TSYM groups in both trials were 100% and 40%, respectively. In contrast, the HBZF group exhibited mild depression and anorexia and recovered within three to five days, which elicited only a minor retardation in growth.

3.4. PRRSV viremia titer

Nucleic acid of PRRSV detected in sera from all challenged pigs reached peak values at 7 and 14 DPC for TSYM and HBZF, respectively. The peak genomic numbers of PRRSV from both TSYM groups (7.1 ± 0.3 and 6.4 ± 3.3 copies/μL) were approximately 60–300 times greater than the HBZF group (4.6 ± 0.2), which was a statistically significant level ($P < 0.05$, Fig. 3D).

3.5. Pathological examination after challenge with PRRSV

Pigs challenged with TSYM-strain PRRSV showed obvious cyanosis of body extremities (Fig. 4A). Lesions caused by secondary infection, including suppurative bronchopneumonia, polyserositis, arthritis and meningitis, were recognized in the TSYM groups (Table 1). Pulmonary lesions of TSYM-challenged pigs revealed a diffuse and meaty texture (Fig. 4C and Table 1). The microscopic scores also illustrate moderate to severe interstitial pneumonia caused by TSYM-PRRSV, which was characterized by type II pneumocyte hyperplasia and accumulation of cellular debris clumps in alveolar spaces (Fig. 4F and G and Table 1). Moreover, perivascular cuffing with mononuclear cells and gliosis in the cerebellum (Fig. 4H), as well as petechial hemorrhage in the lungs and kidneys were noted in some pigs. Disseminated micro-thrombi were also observed in the vessels of the ears (Fig. 4I). The HBZF-PRRSV challenged pigs revealed milder interstitial pneumonia without pathological evidence of secondary infections (Fig. 4B and E).

3.6. In vitro PRRSV inoculation on PAM

The results of the *in vitro* study in flow cytometry analysis indicated the clinically virulent TSYM-strain infected more than 20% of macrophages at an early time point and maintained high infection rates within 24 h, which was significantly higher than the clinically low virulent HBZF strain at each time point (Fig. 5A). The relative viability of PAM declined and reached a statistically significant level with less

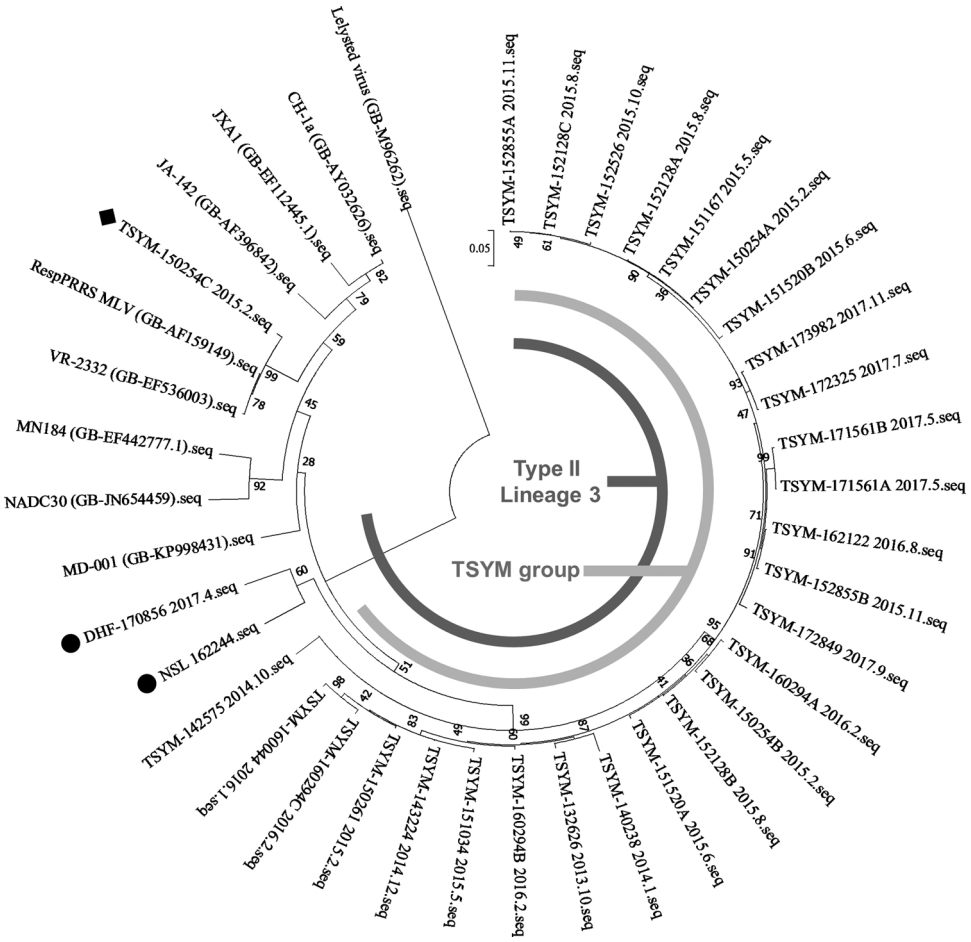

Fig. 2. ORF5-based phylogenetic tree of PRRSV surveillance in Farm TSYM from 2013 to 2017. The representative strains were referred from GenBank, NCBI. Sequences marked with circles indicate PRRSV strains isolated from pigs imported from other farms within two days. Sequences marked with a diamond shared a 99.3% identity with the MLV vaccine strain.

than 50% viability compared to the mock control at 24 h post-inoculation (HPI, Fig. 5B). In contrast, no significant difference between the low virulent strain inoculation and mock control was observed until 36 h.

3.7. Cytokine mRNA quantification

As shown in Fig. 6, normalized mRNA gene expression indicated an up-regulation of interleukin-10 (IL-10) and tumor necrosis factor-α (TNF-α) after PRRSV inoculation, especially with clinically virulent PRRSV TSYM strain inoculation. The level of mRNA accumulation of IL-10 and TNF-α raised up to 23- and 19-fold by 24 HPI compared to that of the mock control group. Up-regulation of IL-4 and IL-12 cytokine mRNA was noted at 24 HPI in the TSYM-group.

4. Discussion

The comparably high virulence strain, PRRSV TSYM isolate, was first noticed due to the sudden increase in mortality of nursery pigs in the PRRSV endemic farm. Most pigs showed post weaning respiratory disorders characterized by severe interstitial pneumonia complicated with secondary infections. The virulent strain subsequently circulated in the farm and became prevalent for years. Based on long-term phylogenic surveillance, we recognized several other strains of PRRSV that had also been carried into the same population. However, the virulent TSYM strain maintained the predominant position within this farm for years. It is similar to the HP-PRRS prevalence pattern, which had been known to have much greater abilities in viral replication than classical strains [15]. Since the outbreak of HP-PRRS, the epidemic immediately expanded to most pig farms in China and replaced the conventional strains to be the most prevalent PRRSV strain during the next several years [16]. This implied that the TSYM-strain may possess higher virulence than other field isolates in this farm.

In our previous experience, most Taiwanese PRRSV isolates elicit only mild, short-lasting clinical signs in healthy pigs under well-controlled housing conditions, as was seen in the control group of HBZF used in this study. However, the TSYM isolate triggers a considerable level of severity in terms of clinical signs, which are characterized by prolonged high fever, labored breathing and even mortalities in both five and eight week-old healthy pigs. Lesions associated with high virulence, such as proliferative necrotizing pneumonia, nonsuppurative encephalitis and petechial hemorrhage in the kidneys, are frequently observed in TSYM-challenged pigs [10,17]. Some previous studies have proven that virulent PRRSV strains possess higher replication efficiency, which could lead to a consequently higher viremia titer [18]. In this study, the TSYM isolates triggered higher infection rates under *in vitro* experiments by flow cytometry analysis and also elicited a 100-fold higher viremia titer than HBZF in pig challenge studies. Moreover, with peak titers of 6.7–7.7 logs μL^{-1} serum after challenge with TSYM isolates, it displayed a level comparable with HP-PRRSV [10,15].

As primary target cell of PRRSV infection, the cytokines produced by PAM are important for the consequential development of pathogenesis and immune responses. IL-10 has been broadly reported to play a vital role in PRRSV induced immune dysfunction. Up-regulation of IL-10 could suppress the cell-mediated immunity and shift immune responses to Th2 bias. Together with glycan shields and decoyed epitopes

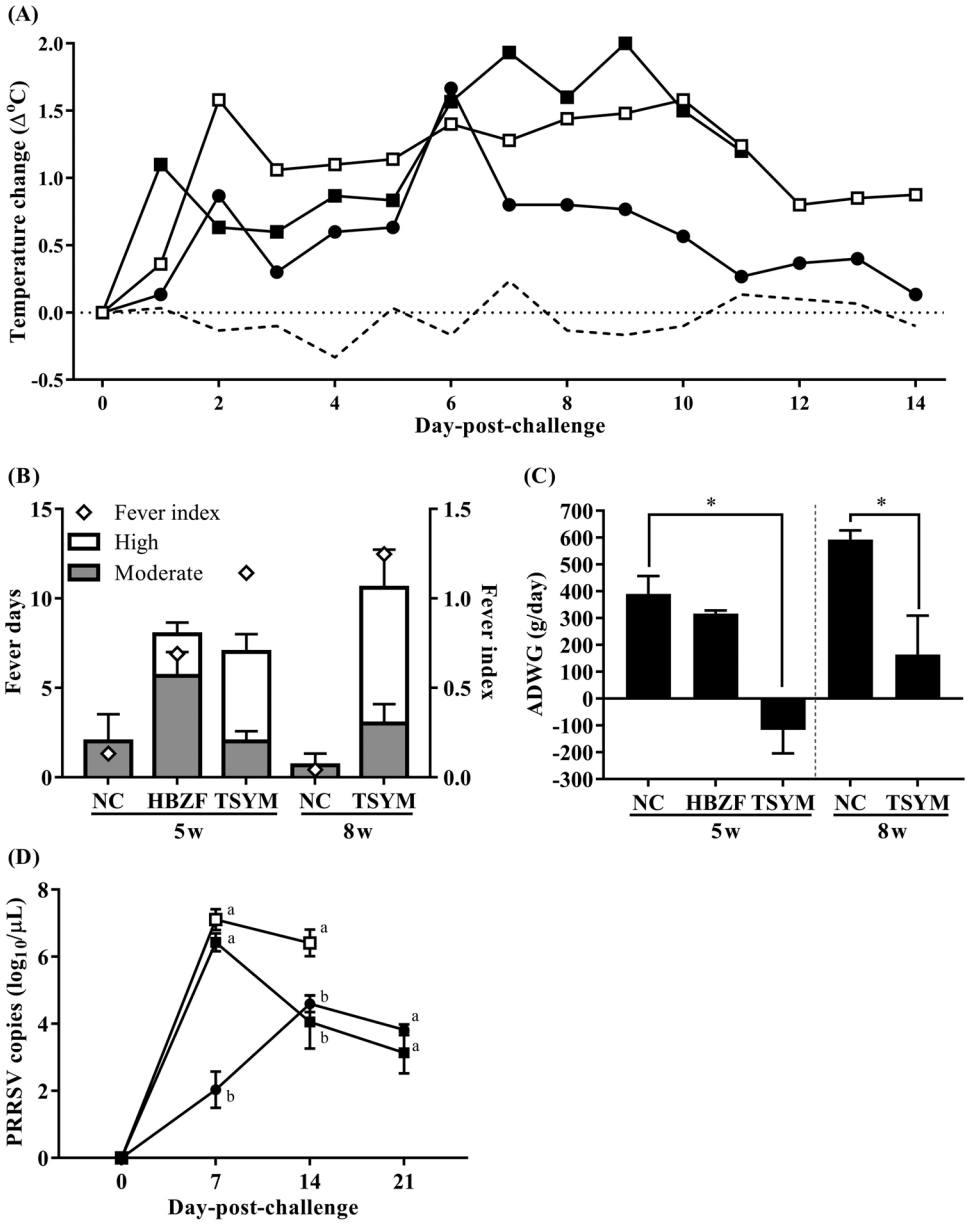

Fig. 3. The changes of body temperature of the mock control (——), HBZF (●) and TSYM (■ for trial I and □ for trial II) groups after PRRSV challenge (A). The basal body temperature was calculated by averaged pre-challenge temperatures (dotted). Changes greater than 0.5 and 1.0 ℃ were respectively defined as moderate and high fever and calculated for fever indexes (◇) (B). The average daily weight gains after challenge with the mock, HBZF and TSYM isolates in trial I and II are summarized (C). The PRRSV viremia titers of HBZF (●) and TSYM (■ for trial I and □ for trial II) groups after PRRSV challenge (D). The calculated data are present as mean ± SE and statistical significances ($P < 0.05$) are indicated with an asterisk (*) or alphabetical letters for different significant levels.

of viral particles, these immunopathological mechanisms eventually lead to weak and delayed adaptive immune responses and viral escaping [19]. Similar conditions were also observed in our *in vitro* studies, in particular, the significant up-regulation of IL-10 in early infection and comparable silence and late onset of IL-1β and IL-12, especially in those inoculated with virulent isolates. Both IL-10 and TNF-α are apoptogenic cytokines that are hypothesized to be associated with bystander death and contribute to the pathogenicity of PRRSV [20]. The considerable rise of both cytokines in the TSYM group might be associated with the apparent PAM death in the viability assay, and the notable necrotizing pneumonia lesions in the TSYM-challenged pigs in this study, which has also been conclusively correlated in other virulent PRRSV strains [21]. The cumulative effects of cell death and dysfunction in cytokine regulation could contribute to the high prevalence of opportunistic infection observed in naturally infected and challenged pigs.

5. Conclusions

Since the first outbreak of PRRSV in 1993, Taiwanese PRRSV strains have undergone evolution and localized in Taiwan for decades, leading to a divergence from the major prevalent PRRSV strains in the rest of the world [12]. This study provides information about a comparably high virulence strain in Taiwan and outlines the appearance in both farm and laboratory levels. The TSYM-strain elicited severe clinical signs and lesions in healthy pigs. This strain also triggers a high viremia titer that is close to that of HP-PRRS. This virulent strain may be more suitable to be used to evaluate the protective potencies of vaccines used in Taiwan or for studies for viral virulence factors. In addition, gaps also remain in the epidemiological status and more data is needed to understand the dynamics of virulent PRRSV strains in Taiwan.

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Conflict of interest statement

The authors declare no conflict of interest.

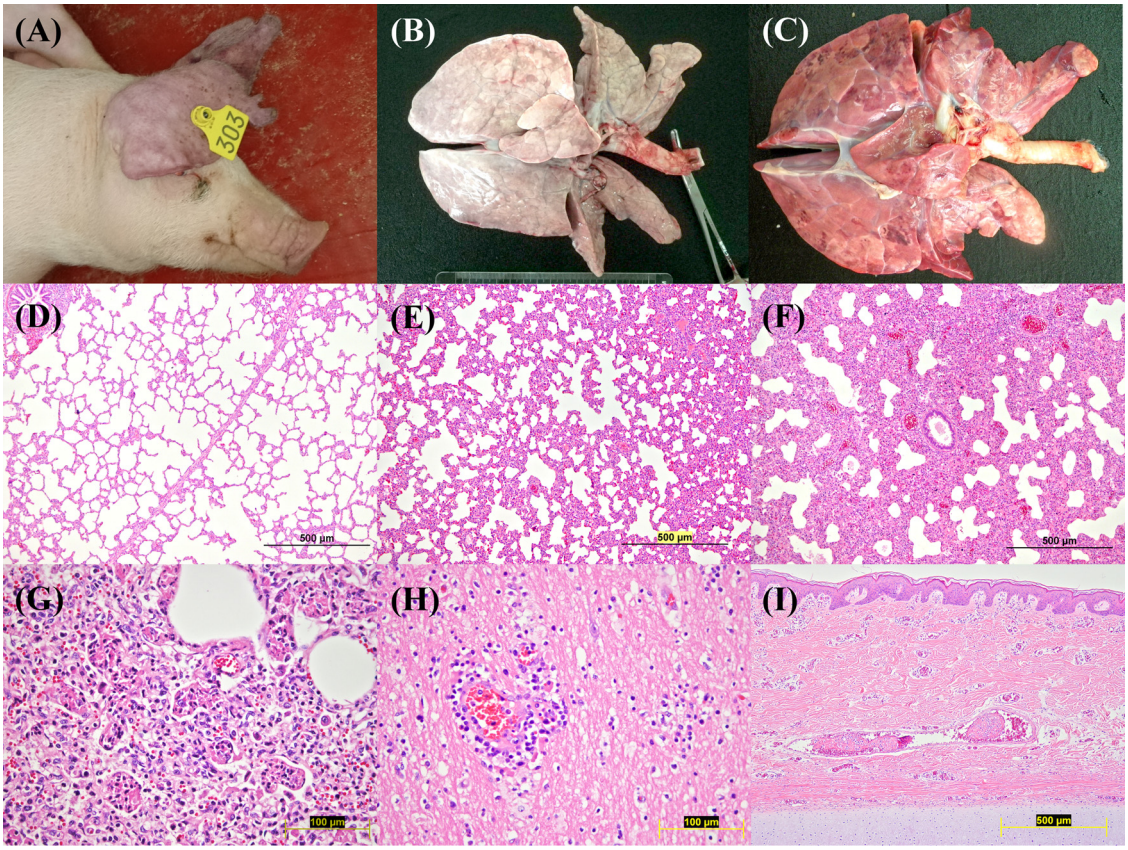

Fig. 4. Macro- and microscopic examination of PRRSV challenged pigs at 21 DPC. Cyanosis of the ears and body extremities were observed in the TSYM-challenged pigs (A). Mild and moderate to severe consolidation and flushing of the lungs were noted in the HBZF (B) and TSYM groups (C). Different levels of septal thickness were recorded in the unchallenged (D), HBZF (E) and TSYM groups (F). The diagnosis of proliferative necrotic pneumonia was made based on the profound amount of cellular debris accumulated in the alveolar spaces in the TSYM group (G). Perivascular cuffing with mononuclear cells and gliosis in the cerebrum were also noted in pigs challenged with TSYM–isolates (H). Disseminated occupation with micro-thrombi were observed in the vessels of the ears (I).

Table 1
Pathological records of nursery pigs challenged with HBZF or TSYM strains of PRRSV.

Group	Age (week)	SBP[c]	Serositis[b]	IP score[a,c]		PNP[c]	Mortality
				Macroscopic (%)	Microscopic		
Trial I							
Mock	5	0/2[d]	0/2	3.5 ± 3.5	0.95 ± 0.55	0/2	0/3 (0%)
HBZF	5	0/3	0/3	42.7 ± 7.9	1.97 ± 0.38	1/3	0/3 (0%)
TSYM	5	1/3	3/3	94.0 ± 5.5	2.43 ± 0.26	3/3	3/3 (100%)
Trial II							
Mock	8	0/5	0/5	8.4 ± 1.8	0.07 ± 0.07	0/5	0/5 (0%)
TSYM	8	2/5	1/5	60.6 ± 15.6	2.22 ± 0.58	2/5	2/5 (40%)

[a] Data were indicated as mean ± standard error (SE).
[b] Pleuritis, pericarditis, peritonitis, arthritis and meningitis were included.
[c] Abbreviation: SBP, suppurative bronchopneumonia; IP, interstitial pneumonia; PNP, proliferative necrotizing pneumonia.
[d] Number of positive/number of samples.

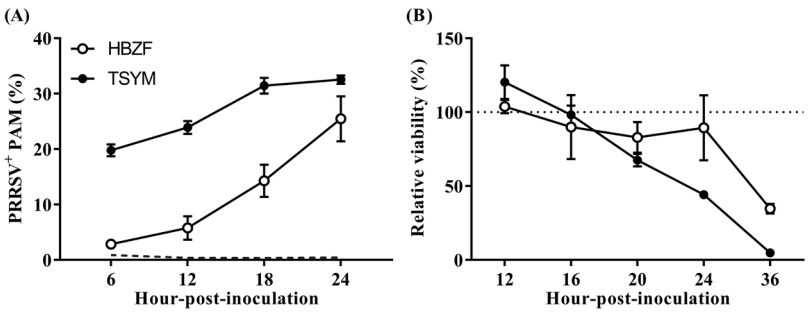

Fig. 5. The PRRSV titers (A) and viabilities (B) of HBZF and TSYM-isolate inoculated PAM. The values are calculated by measuring PI^- and SDOW-17-$FITC^+$ PAM populations on flow cytometry after different inoculation intervals. The mock controls are presented as a dashed line in PRRSV IFA assay and basal value for viability assay. All data are indicated as mean ± SE of triplicated experiments.

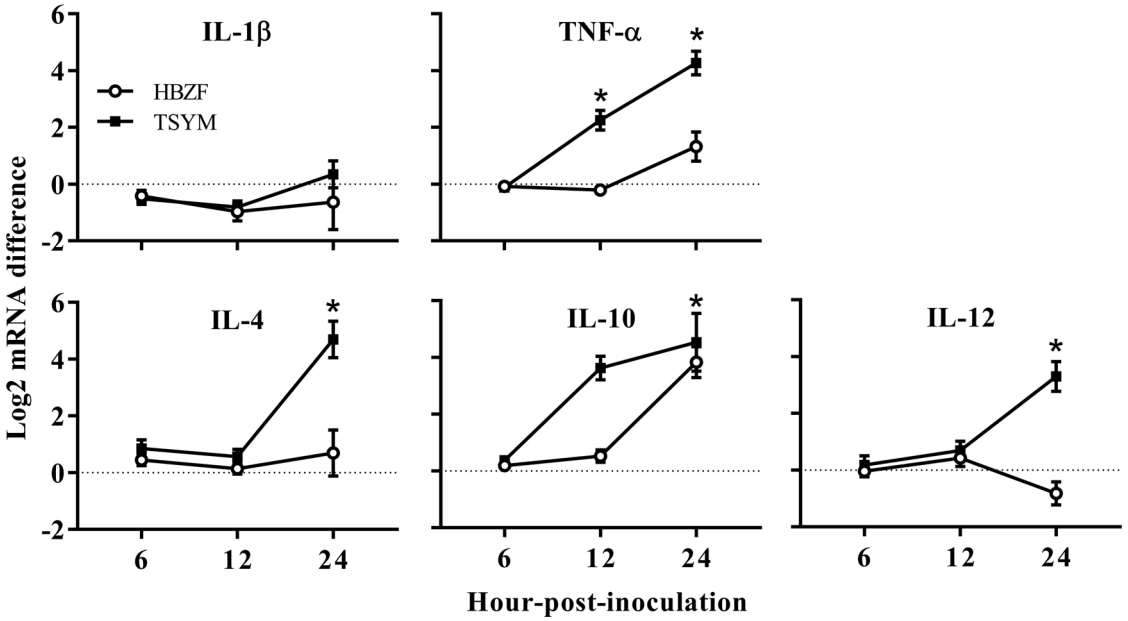

Fig. 6. Cytokine mRNA profile of HBZF- (○) and TSYM-isolates (■) inoculated PAMs. The mock controls are used as a basal line (dotted line). The relative mRNA quantities are calculated by $2^{-\Delta\Delta Ct}$ method and indicated as mean ± SE of triplicated experiments. Statistical significances ($P < 0.05$) compared to the mock controls are indicated with an asterisk (*).

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