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The phosphorylation of the N protein could affect PRRSV virulence *in vivo*

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A B S T R A C T

The porcine respiratory and reproductive syndrome virus (PRRSV) nucleocapsid (N) protein is a multiphosphorylated protein.It has been proved that the phosphorylation of N protein could regulate the growth ability of PRRSV in Marc-145 cells. However, further investigation is needed to determine whether phosphorylation of the N protein could affect PRRSV virulence in piglets. In this study, we confirmed that the mutations could impair PRRSV replication ability in porcine primary macrophages (PAMs) as they did in Marc-145 cells. The animal experiments suggested that the pathogenicity of the mutated virus (A105-120) was significantly reduced compared with parent strain (XH-GD). Our results suggested that the phosphorylation of the N protein contributes to virus replication and virulence. This study is the first to identify a specific modification involved in PRRSV pathogenicity. Mutation of PTMs sites is also a novel way to attenuate PRRSV virulence. The mutations could be a marker in a vaccine. In conclusion, our study will improve our understanding of the molecular mechanisms of PRRSV pathogenicity.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS), one of the most important disease in pig industry, is characterized by respiratory distress in piglets and reproductive failure in pregnant sow. It is caused by porcine reproductive and respiratory syndrome virus (PRRSV) (Cui et al., 2015).In 1987, PRRSV were firstly reported in North America, since then, the virus were found around world (Rosendal et al., 2014). In 2006, high pathogenicity (HP) PRRSV was reported in China (Bai et al., 2018). Currently, PRRSV has become one of the most important pathogens for the global swine industry (Chen et al., 2016).

The PRRSV is a single positive-strand RNA virus, the genome length is approximately 15 kb (Yu et al., 2018).It contains at least 16 non-structural proteins and 8 structural proteins (Xu et al., 2018a; 2018b).According to the genetic variation and differences of the antigen, PRRSV can be divided into two types, including the European type (PRRSV-1) and the North American type (PRRSV-2) (Xie et al., 2017).The PRRSV-1 and PRRSV-2 share only 60% nucleotide sequence

homology (Nan et al., 2017).

Post-translation modifications (PTM) occur during or after protein biosynthesis (Knorre et al., 2009). Including phosphorylation, acetylation, glycosylation, SUMOylation, many proteins contain PTM, and the protein functions can be regulated by those modifications (Xu et al., 2018a; 2018b; Yang et al., 2019). For example, the SUMOylation of AR (androgen receptor) can change mouse bone mass (Wu et al., 2019), and the phosphorylation of HSV-1 UL51 can regulate HSV-1 replication and pathogenicity (Kato et al., 2018). Among the various kinds of modifications, phosphorylation has been one of the most extensively studied subjects (Chen et al., 2018a; 2018b).

The N protein is the most abundant protein in PRRSV. It interacts with viral RNA to form nucleocapsids and participates in virion assembly (Snijder et al., 2013). N also has been proved to regulate the host immune response (Yu et al., 2017).Further study has suggested that the N protein is a multiphosphorylation protein (Wootton et al., 2002). It contains at least two phosphorylation modification sites. Mutation of those sites could impair the viral replication ability in

Marc-145 cells (Chen et al., 2018a; 2018b). However, whether the mutation could impair PRRSV virulence to piglets requires further investigation.In this study, we found that the mutations could impair PRRSV replication ability in porcine primary macrophages (PAMs). The animal experiments indicated that the pathogenicity of the mutated virus (A105-120) was significantly lower than parent strain (XH-GD). Our results suggested that the phosphorylation of the N protein contributes to virus replication and virulence.The results are also beneficial to find a novel way to attenuate the virus.

2. Materials and methods

2.1. Virus and cells

The porcine alveolar macrophages (PAMs) and Marc-145 cells were maintained in DMEM (Gibco, USA) at 37 °C in 5% CO_2. The PRRSV-2 strain XH-GD (GenBank no. EU624117.1) and mutated viruses (A105, A120 and A105-120) were used in the virus infection studies(Chen et al., 2018a; 2018b).

2.2. Multistep growth curve in PAMs

A previous report showed that the mutation could affect the viral growth efficiency in Marc-145 cells. To further investigate the replication efficiency of the mutated virus in PAMs, the cells were cultured in 6-well plates, and each well contained 1×10^5 cells. After culturing for 24 h, a viral MOI of 0.1 was used to infect the PAMs. The supernatant were collected at particular time points (12, 24, 36 and 48 hpi). The virus titer or viral Nsp9 gene level of the supernatant were calculated in Marc-145 cells as in previous reports (Chen et al., 2018a; 2018b).

2.3. Animal experiment

Eighteen 4-week-old piglets were obtained from Guangxi State Farms, which is known to be free of PRRSV. All piglets were confirmed to be negative for PRV, CSFV, SIV, PCV2 and PRRSV by commercial IDEXX ELISA kits and RT-PCR. The piglets were fed in the Experimental Animal Centre of South China Agricultural University.

According to the design, the piglets were randomly divided into three groups (negative, A105-120 and XH-GD, n = 6). All piglets were infected with 2 mL 10^6 $TCID_{50}$/mL respective virus (A105-120 and XH-GD) by trachea injection. The negative group piglets were inoculated with the same dose of DMEM. Clinical signs of every piglet were recorded. Blood samples were collected at 0, 3, 7, 10, 14 and 21 days post-inoculation (dpi). The weight of every piglet was recorded every three days. At 7 and 14 dpi, one piglet from each group was randomly selected to detect the viral titre of the lung and lymph gland. According to the operation manual, the PRRSV antibody level was detected by commercial ELISA (IDEXX, USA). All surviving piglets were euthanized at 21 dpi, and the lungs were collected for histological examination.

2.4. Real-time PCR quantification of viral RNA copies in the serum and organs from infected piglets

To study the virus replication ability *in vivo*, the serum was collected at particular time points (0, 3, 7, 10, 14 and 21 dpi). The lung and lymph gland were collected at 7, 14 and 21 dpi. One gram organ sample or 100 μL serum was used to extract the viral RNA. The method was used to extract the RNA and synthesize the cDNA as same as previous reports (Xie et al., 2014). Real-time PCR was carried out on a CFX96™ real-time system (Bio-Rad, USA). The Nsp9 primes were used in this study (qRT-Nsp9-F:CCTGCAATTGTCCGCTGGTTTG; qRT-Nsp9-R:GAC GACAGGCCACCTCTCTTAG; qRT-Nsp9-probe:FAM-ACTGCTGCCACGA TTTACTGGTCACGCAGT-BHQ1).

According to the *Premix Ex Taq*™(Probe qPCR) (Takara, Japan)

guidebook, the qRT-PCR program was 95 °C for 30 s followed by 40 cycles at 95 °C for 5 s and 60 °C for 30 s. For each assay, a standard serially diluted PRRSV strain XH-GD (10^0-10^8 $TCID_{50}$/mL) was used to generate a standard curve (slope = − 3.261; R^2 = 0.991) (Liu et al., 2014; Zhao et al., 2018).

2.5. Data analysis

Statistical analyses were performed using SPSS software (version 21.0). Data collected from three independent experiments were analysed as the means ± standard deviations (SD). The differences among the groups were calculated by Tukey's test. A *p* value < 0.05 was considered statistically significant.

2.6. Ethics statements

The animal experiments in this study were approved by the Laboratory Animal Committee of South China Agricultural University (No. 2018D102). The experiments were carried out in accordance with animal ethics guidelines and approved protocols.

3. Results

3.1. The identified phosphorylation modification of the N protein affected viral growth in PAMs

A previous report showed that the phosphorylation of the N protein could impair the viral replication ability in Marc-145 cells(Chen et al., 2018a; 2018b). However, since PAMs cells play an important role during the PRRSV infection(Burkard et al., 2017), we questioned whether the growth curve of viruses in PAMs was similar to that in Marc-145 cells. In this study, viruses were incubated with the PAMs at an MOI of 0.1. Viral titres were calculated by the Reed-Muench method. As shown in Fig. 1, the titre of XH-GD was always higher than that of the other mutated viruses at 12–36 hpi. XH-GD reached the maximum titre at 36 hpi, which is faster than the other viruses. Compared with XH-GD, A105 and A120, A105-120 showed lower viral growth at 24–36 hpi. There were significant differences between XH-GD and A105-120 at 24 hpi and 36 hpi *(P < 0.01)* (Fig. 1A). The Nsp9 gene real-time PCR results were similar with growth curve (Fig. 1B). This finding suggested that phosphorylation modification of the N protein could regulate viral growth in PAMs as it did in Marc-145 cells.

3.2. Modification of the N protein affected the pathogenicity of PRRSV in piglets

We examined the effects of phosphorylation modification of the N protein on PRRSV infection *in vivo*. Eighteen 4-week-old piglets were infected with XH-GD, A105-120 or DMEM by trachea injection. The mortality and clinical symptoms were monitored for 21 days post-infection. We found that the mutation could significantly relieve the clinical symptoms. As shown in Table 1, compared with the piglets infected with XH-GD, the piglets infected with A105-120 displayed a better clinical response, including cough, appetite, mental state, state of dorsal septa and skin erubescence.

HP-PRRSV could lead pigs to have high fever (> 40.5 °C)(Chen et al., 2016). The mean rectal temperature of piglets in the XH-GD group was always higher than that of those in the A105-A120 group after inoculation with the respective virus. The mean temperature for the XH-GD group was higher than 40.5 °C from 7 to 13 dpi. However, the mean temperature for the other groups was always lower than 40.5 °C. The temperature of pigs infected with XH-GD was significantly higher than that of the A105-120 group at 10–12 dpi and 16 dpi (Fig. 2A).

The average weight in each group was calculated. At the first 3 dpi, there were no significant differences among the three groups. However,

A

B
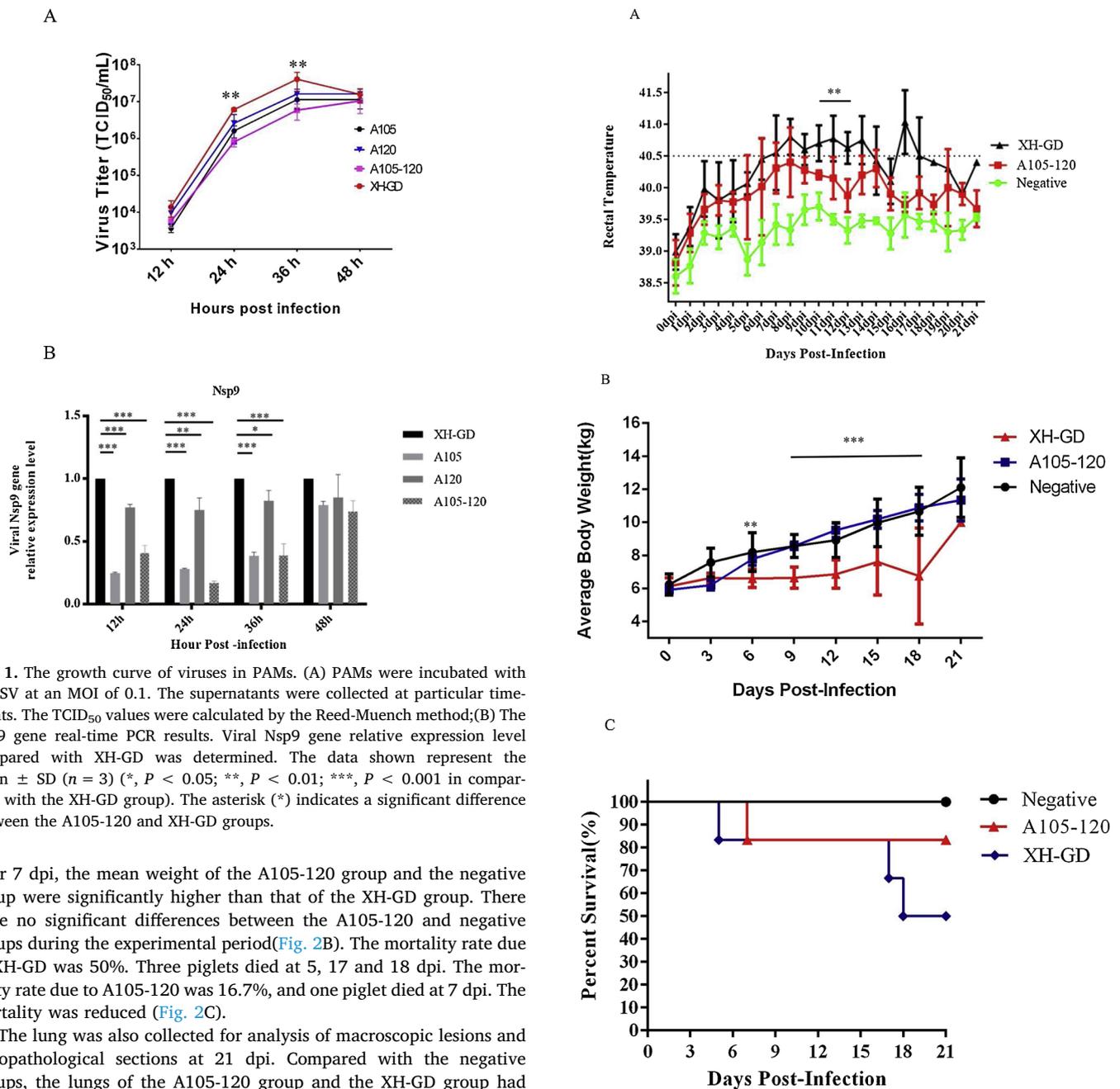
Fig. 1. The growth curve of viruses in PAMs. (A) PAMs were incubated with PRRSV at an MOI of 0.1. The supernatants were collected at particular time-points. The $TCID_{50}$ values were calculated by the Reed-Muench method;(B) The Nsp9 gene real-time PCR results. Viral Nsp9 gene relative expression level compared with XH-GD was determined. The data shown represent the mean $\pm$ SD ($n = 3$) (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ in comparison with the XH-GD group). The asterisk (*) indicates a significant difference between the A105-120 and XH-GD groups.

after 7 dpi, the mean weight of the A105-120 group and the negative group were significantly higher than that of the XH-GD group. There were no significant differences between the A105-120 and negative groups during the experimental period(Fig. 2B). The mortality rate due to XH-GD was 50%. Three piglets died at 5, 17 and 18 dpi. The mortality rate due to A105-120 was 16.7%, and one piglet died at 7 dpi. The mortality was reduced (Fig. 2C).

The lung was also collected for analysis of macroscopic lesions and histopathological sections at 21 dpi. Compared with the negative groups, the lungs of the A105-120 group and the XH-GD group had significantly widened alveolar interstitial spaces. However, the lungs of pigs in the A105-120 group had fewer lesions and histopathological changes than those in the XH-GD group (Fig. 3).

In summary, the mutated virus still maintains pathogenicity. However, compared with parent virus, the mutations led to alleviated clinical response, temperature response, and decreased mortality and did not affect weight gain of the inoculated piglets.

A

B

C

Fig. 2. The clinical response of different groups. (A) The rectal temperature. The value is presented as the mean $\pm$ standard deviation (shown by error bars). (B) The weight of different groups. (C) The survival curves of different groups (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

3.3. The phosphorylation of the N protein affected PRRSV replication ability in piglets

The serum, lung and lymph gland were used to detect the viral loads

Table 1
The clinical symptoms of different groups.

	High fever(> 40.5 °C)	Mental state	weigh loss	Anorexia	Dyspnea	Mortality
XH-GD	6[a]	5	5	5	4	50.00%
A105-120	2	1	0	1	0	16.67%
Negative	0	0	0	0	0	0.00%

[a] The number refers to the number of piglets that had the symptom during the experiment.
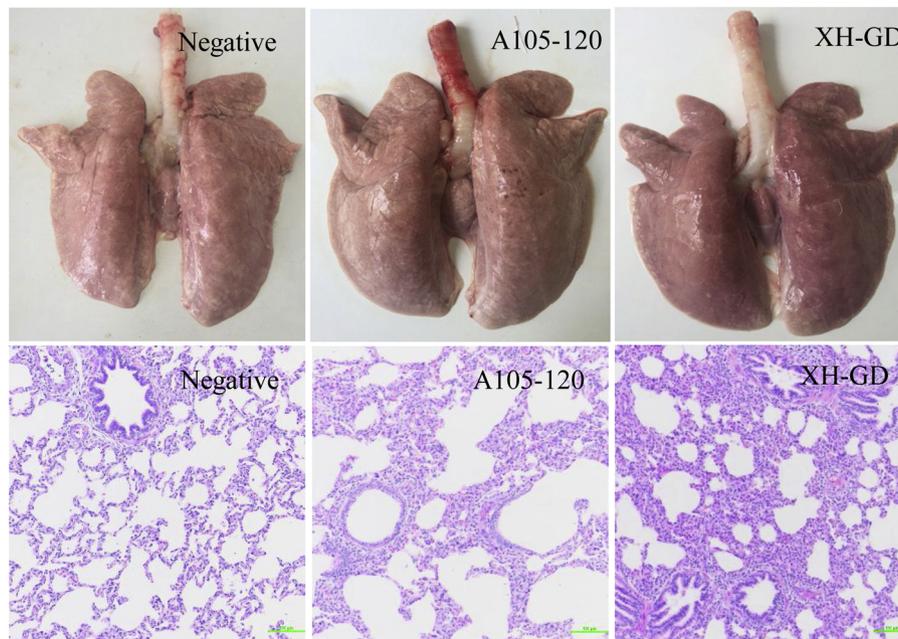
Fig. 3. Macroscopic lung lesions and microscopic lung lesions of piglets.

(Han et al., 2017). In this experiment, the serum of every piglet was collected at 3, 7, 10, 14 and 21 dpi. At 7, 14 and 21 dpi, one piglet from each group was randomly selected and euthanized. The lung and lymph gland were collected. We determined the virus copy numbers in the serum, lung and lymph gland by qRT-PCR. In serum, the viral load reached a peak at 10 dpi. At 3 dpi and 7 dpi, there were significant differences between the two groups ($P < 0.001$)(Fig. 4A). In the lung, the peak appeared at 14 dpi. Regardless of the lung or lymph gland, at 7 dpi and 14 dpi, the viral load of XH-GD were always higher than that of A105-120, and there were significant differences between the two groups ($P < 0.001$) (Fig. 4B, C). The difference between the two groups decreased at 21 dpi. The replication efficiency of A105-120 was lower than that of the parent virus *in vivo*.

Then, we detected the antibody level. We collected serum at 3, 7, 14 and 21 dpi. Using the commercial ELISA kit to detect the antibody level against N protein, we found that there were no significant differences between the XH-GD and A105-120 groups in antibody levels (Fig. 5). The mutations did not affect the antigenicity of the N protein or the immune response of piglets.

4. Discussion

PTMs occurs during or after protein biosynthesis (Knorre et al., 2009). It plays an important role in many biological processes, such as protein degradation, regulation of gene expression and protein function state. PTMs have many types, including ubiquitination, methylation, glycosylation and phosphorylation. Mutated modification sites may be directly involved in the cell or viral life cycle (Song and Brady, 2015; Wu et al., 2019). For example, phosphorylation of CA/P24 could affect the HIV reverse transcription process (Chen et al., 2018a; 2018b). Glycosylation of ECP (the human eosinophil cationic protein) regulates its antimicrobial ability (Salazar et al., 2014).

There are many pathogens that have serious effects on society in the *Nidovirales* order, such as MEAR, SARS, IBV, PEDV and PRRSV (de Wilde et al., 2018). The N proteins of those viruses have a common origin (Yu et al., 2006). Therefore, investigating the function of the N protein of PRRSV will help to understand the role of the N protein in the *Nidovirales* life cycle. The N protein of PRRSV, a phosphorylated protein, is important for the viral life cycle. A previous study showed that mutated phosphorylation modification sites could impair growth in

vitro (Chen et al., 2018a; 2018b). Since PAMs cells were the target cell of PRRSV in the lung (Burkard et al., 2017; Shabir et al., 2018), they were used to confirm the effect of the mutations. The growth curve results showed that the mutations could also regulate the replication ability in PAMs. We confirmed once again that phosphorylation modification of the N protein could regulate PRRSV replication.

Phosphorylation has been shown to regulate the pathogens growth and virulence (Albataineh and Kadosh, 2016). However, whether phosphorylation modification of the N protein could affect PRRSV virulence in piglets still needs to be investigated. In this study, eighteen piglets were divided into three groups, and their clinical performance was recorded. The animal experiment showed that the mortality of the A105-120 group was lower than that of the XH-GD group, and the weight of piglets was significantly higher in the mutant virus group than that of those in the parent virus group during 3 dpi-18 dpi.

For the other clinical symptoms, the A105-120 group was significantly less severe than the XH-GD group. These results indicate that phosphorylation of the N protein could impair XH-GD virulence. However, we also found that the mutated virus (A105-120) could also cause death in piglets. The rectal temperature, the macroscopic lesions and the histopathological sections results show that the mutated virus still causes tissue damage, suggesting that the virus still maintains some pathogenicity.

Since mutations could impair the growth *in vitro*, we suspected that the replication ability *in vivo* would decrease as *in vitro*. At the particular time points, we selected one piglet and used real-time PCR to detect the virus load in the serum, lung and lymph gland. In serum, the viral load of A105-120 was significantly lower than that of XH-GD at 3 dpi and 7 dpi. In the organs, the viral load of XH-GD was significantly higher than that of A105-120 at 7 dpi and 14 dpi. These results indicate that phosphorylation modification of the N protein is vital for virus replication *in vivo*. The ELISA results showed that the mutations did not affect the antigenicity of the N protein or the virus.

Currently, attenuated vaccines are widely used on farms (Zhou et al., 2018). They have become a powerful tool to protect pigs from PRRSV. However, the most of vaccines do not contain stable markers. It is difficult to distinguish the vaccines from the wild-type virus. The emergence of recombinant viruses has exacerbated this problem (Liu et al., 2017; Zhou et al., 2018, 2015). The N protein is the most conserved protein in PRRSV, and few sites have changed during passaging

A

Serum

B

Lung

C
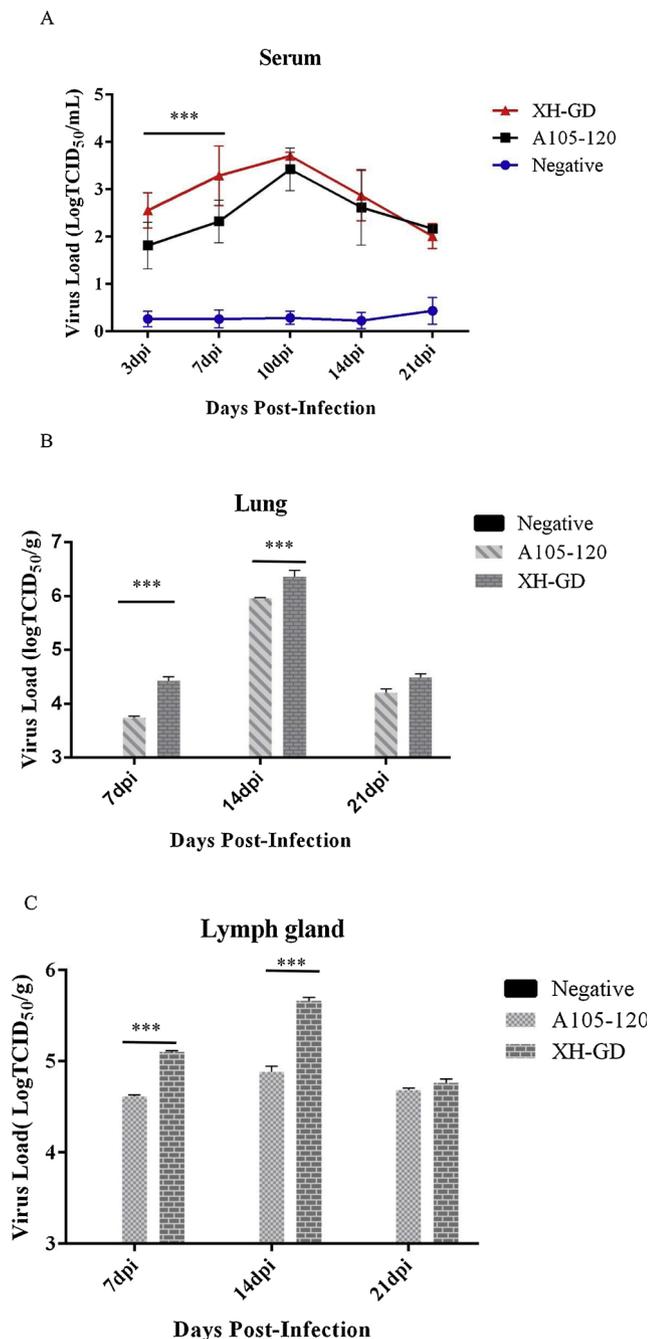
Lymph gland

Fig. 4. The viral load of the organs and serum at particular timepoints. The viral load was quantified by real-time PCR. (A) The viral load in lung represented as viral RNA copies per gram; (B) the viral load in one gram lymph gland; (C) The viral load in serum per millilitre. The data shown represent the mean $\pm$ SD ($n = 3$) (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

in vitro (Chen et al., 2016; Liu et al., 2014). There are no reports showing that the ORF7 gene (encoding the N protein) could exchange between different viruses. Therefore, we speculated that the mutations could be a vaccine marker.

In summary, our study confirmed that phosphorylation of the N protein could impair virus growth and affect virulence in piglets. The mutation of PTM sites is a novel way to attenuate viruses. The mutations may be a marker to distinguish a vaccine from a wild-type virus. Our study will help to elucidate the mechanisms of PRRSV replication and pathogenicity. Since the N protein structures are similar, our research could also be the model to study other members of the *Arterivirus* or *Coronavirus* genera.
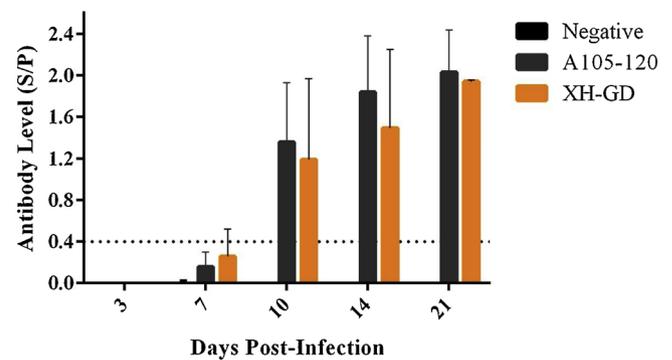
Fig. 5. The anti-N protein antibody levels at different timepoints. A value ≥ 0.4 was considered positive.

Author contributions

Designed the experiments: YC, ZY. Performed the experiments: YC, YQ, QL. Performed the animal experiments: YC, ZY, HY, FW, XH, QL, CJ, JH, QD, YL, MC, SH. Analysed the data: SH. Wrote the manuscript: YC. Revised the manuscript: GZ, CM.

Conflict of interest

The authors declare that they have no conflicts of interest.

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