RAPID COMMUNICATION

Outbreak Investigation of NADC30-Like PRRSV in South-East China

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Summary

Epidemiological outbreak investigations were conducted on NADC30-like porcine reproductive and respiratory syndrome virus (PRRSV) to investigate the prevalence of the disease in south-east China in 2015. Two more provinces were found to have NADC30-like PRRSV circulating besides previously reported six provinces. Phylogenetic analysis showed that these virus isolates were clustered in an independent branch and shared high nucleotide similarity to NADC30, a type 2 PRRSV that has been isolated in the United States of America in 2008. One NADC30-like PRRSV strain from Henan province was successfully isolated on porcine alveolar macrophages and was tested on 6-week-old specific pathogen-free pigs for pathogenic study. The virus-inoculated pigs showed typical PRRSV clinical symptoms, but all pigs survived throughout the study with a period of 14 days. At necropsy, the lungs of infected pigs developed PRRSV-specific interstitial pneumonia, and virus antigen was detected in lung samples. Therefore, our results indicated NADC30-like PRRSV has widely spread in China and could cause clinical disease on pigs.

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically important pig diseases that cause huge economic losses to the global swine industry. The causative agent porcine reproductive and respiratory syndrome virus (PRRSV) belongs to the Order Nidovirales, family Arteriviridae (Conzelmann et al., 1993). PRRSV can be divided into European genotype 1 and North American genotype 2 with VR2332 and Lelystad as prototypical strains, respectively. VR2332 was firstly isolated from infected pigs in the United States of America in 1992, which caused clinical respiratory syndrome with low mortality (Collins et al., 1992). However, PRRSV is notorious for the high incidences of mutation and recombination rates, which lead to the emergence of new circulating strains with different levels of virulence. A good example is the outbreak of a highly pathogenic PRRSV (HP-PRRSV) in China, which was characterized by high morbidity and mortality with all ages of pigs and resulted in the loss of one million pigs in 2006 (Tian et al., 2007). Since 2014, several field isolates of PRRSV had a very unique genetic background and showed the highest nucleotide similarity to a group represented by NADC30, a type 2 PRRSV featured by clinical fever with low mortality that has been isolated in the United States of America in 2008 (Brockmeier et al., 2012; Zhao et al., 2015; Zhou et al., 2015a). Accordingly, these PRRSV isolates were designated as NADC30-like PRRSV in China.

Since 2014, several outbreaks of NADC30-like PRRSV were reported in different provinces in north-east and Middle China (Brockmeier et al., 2012; Zhao et al., 2015; Zhou et al., 2015a). The viruses were isolated from vaccinated pigs, which showed clinical respiratory symptoms
indicating the fact that current commercial PRRSV vaccines could not provide complete protection to the circulating NADC30-like PRRSV. The low genome identity of NADC30-like PRRSV with current vaccine strains of PRRSV may explain less cross-protection of commercial vaccines to the currently circulating NADC30-like PRRSV. To investigate the circulation of NADC30-like PRRSV in south-east China where pig breeding stock accounted for more than 70% of total stock in China, a total of 230 serum and tissue samples of diseased pigs from 16 provinces were

Fig. 1. Distribution of NADC30-like PRRSV in China. The provinces involved in this study were indicated by both light and dark shadows except Heilongjiang, Jilin and Zhejiang. Previously reported NADC30-like PRRSV-positive provinces were indicated by dark shadow. The two NADC30-like PRRSV-positive provinces (Henan and Fujian) in this study were shown by an extra star in the dark shadows.

Fig. 2. Phylogenetic analysis of whole genome of PRRSV HNJz15 with other representative PRRSV strains including representative prototype strain VR2332 (GenBank access no. U87392), JXA1 (EF112445), HuN4 (EF635006), CH1a (AY032626), BJ-4 (AF331831), NADC30 (JN654459), HENAN-XINX (KF611905), HENAN-HEB (KJ143621) and JL580 (KR706343). The phylogenetic tree was constructed using a distance-based neighbour-joining method with 1000 bootstrap replicates in MEGA6. Numbers along the branches are bootstrap values. Scale bar indicates nucleotide substitution per site.
collected in 2015. RT-PCR was used for the initial screening of PRRSV-positive samples. All virus-positive samples were next to be used for virus isolation on porcine alveolar macrophages (PAMs). The purified virus was subjected to sequencing for phylogenetic analysis. Finally, one NADC30-like PRRSV isolate HNjz15 was successfully isolated and used to infect 6-week-old pigs for pathogenesis study.

Materials and Methods

A total of 230 clinical samples of diseased pigs (81 serum samples and 149 lung and lymph node samples) were collected from 54 swine farms in 16 provinces in 2015. The animals were sampled under the permission of their respective managers. The animal trial in this study was approved by the Animal Care and Ethics Committee of National Research Center for Veterinary Medicine and conventional animal welfare regulations and standards were taken into account.

Total RNA was extracted from samples using a RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. A conventional RT-PCR with a pair of specific primers that can differentiate NADC30-like, HP-PRRSV and typical PRRSV was used for the initial screening of PRRSV-positive samples. The length of PCR amplicons will be 681 bp in NADC30-like PRRSV-positive samples, 984 bp in HP-PRRSV-positive samples and 1074 bp in typical PRRSV. The primer sequences are F: 5’-TTGATTGGGAT GTTGTGCTTC-3’, R: 5’-CAATGATGGCTTGAGCTG AGT-3’.

The PRRSV-positive samples were inoculated on PAMs for virus isolation as previously described (Zeman et al., 1993). The inoculated cells were maintained at 37°C in a 5% CO₂ atmosphere and were monitored daily for cytopathic effects (CPE). The cultures were harvested when CPE reached in 70% of the cells and were stored at −80°C as the virus stock until use. The virus was purified by plaque assay for every passaging. Total RNA was extracted from cell cultures and was subjected to gene sequencing as described previously (Zhou et al., 2015b). Multiple alignments were performed by the genome of isolated virus as compared with other typical PRRSV sequences that are available in GenBank. Phylogenetic trees were constructed from aligned nucleotide sequences using the neighbour-joining method and were subsequently subjected to bootstrap analysis with 1000 replicates to determine the percentages of reliabilities at each internal node of the tree. The tree was produced by using the MEGA4.1 program.

The 3rd passage of NADC30-like PRRSV HNjz15 (isolated in Henan province) was used for the pathogenesis study. Ten 6-week-old pigs were tested to be PRRSV negative by ELISA and RT-PCR and were divided into two groups. Pigs in above two groups were inoculated intranasally with 2 ml (5 × 10⁴ TCID₅₀/ml) HNjz15 and placebo culture medium, respectively. Clinical signs and rectal temperatures were observed and recorded daily. Blood samples were collected periodically (0, 4, 8 and 14 days post-infection [dpi]) for PRRSV-specific antibody (IDEXX, Westbrook, ME, USA) detection. All pigs were humanly euthanized at 14 dpi. At necropsy, lung samples were fixed in 10% buffered neutral formalin for haematoxylin and eosin (H&E) and immunohistochemistry (IHC) staining as previously described (Tian et al., 2007).

Results and Discussion

The serum and tissue samples were tested for viral RNA using RT-PCR, and the primers were target to PRRSV non-structural protein 2 (nsp2) gene. Among 230 clinical samples of diseased pigs, 17 samples were tested positive for HP-PRRSV and six samples (five samples collected from Henan province and one sample collected from Fujian province) were tested positive for NADC30-like PRRSV. The
positive ratios of HP-PRRSV and NADC30-like PRRSV were 7.4% and 2.6%, respectively. As for the distribution of PRRSV, nine provinces (Henan, Beijing, Hebei, Hubei, Jiangsu, Shandong, Shanxi, Sichuan and Chongqing) were positive for HP-PRRSV, and two provinces (Henan and Fujian) were positive for NADC30-like PRRSV in this study (Fig. 1). Among these provinces, Henan showed positive to both HP-PRRSV and NADC30-like PRRSV. As our epidemiology investigation only covered 16 provinces in south-east China, the previously reported NADC30-like positive six provinces are also indicated in Fig. 1. Therefore, NADC30-like PRRSV has spread to at least eight provinces in China since its first report in 2014.

The six NADC30-like PRRSV-positive PCR products were subjected to gene sequencing, and the results showed that they shared 93.2–95.8% nucleotide similarity with NADC30. The six NADC30-like PRRSVs had three discontinuous deletions in nsp2 as previous reported NADC30-like PRRSVs, and shared 98.5–100% nucleotide similarity with each other. One NADC30-like strain, designated as HNjz15 that was isolated in Henan province, was subjected to genome sequencing. The full length of HNjz15 (GenBank access no. KT945017) was 15019 nucleotides excluding the 3′ poly (A) tails and shared 95.6% and 89.6% nucleotide similarity with NADC30 and HP-PRRSV JXA1 strains. Phylogenetic analysis based on the full-genome sequences of HNjz15 and other reported NADC30-like PRRSV showed that they were more closely related to NADC30 and were clustered into a separate branch, which distinguish themselves from HP-PRRSV cluster represented by JXA1 and HuN4-F114, respectively (Fig. 2). Therefore, based on the unique genetic marker of NADC3-like PRRSVs and genetic divergence with current dominant HP-PRRSV on genome sequences, we propose that NADC30-like PRRSV strains could be introduced into China by importing of breeding pigs from other countries.

To investigate the pathogenesis of NADC30-like PRRSV, HNjz15 was passaged on PAMs for three times. Growth kinetics of HNjz15 and JXA1 was compared on PAM cells and the results showed that there was no significant difference as for virus growth in vitro (Fig. 3a). The plaque sizes of HNjz15 PRRSV were also similar to JXA1 (Fig. 3b). In animal experiment, pigs in two groups were intranasally inoculated with the 3rd passages of HNjz15 (1 × 10^5 TCID50/pig) and culture medium, respectively. The HNjz15-infected pigs started to develop fever (body temperature ≥40°C) at 1 day post-inoculation (dpi) and the fever lasted for a consecutive 12 days. Clinical symptoms including cough, anorexia, shivering and respiratory distress were observed from 3 dpi to 10 dpi before the infected pigs recovered from the disease. By contrast, pigs in control group behaved normal and had no fever throughout the study. Serum samples collected at 8 and 14 dpi were all antibody positive for PRRSV using IDEXX ELISA kit. The virus was re-isolated from the sera of pigs in infected group, and nps2 gene was sequenced to confirm it.

Fig. 4. Lung gross pathology, histopathology and immunohistochemistry staining of HNjz15-infected and control pigs. Typical consolidation in the lung of HNjz15-infected pigs (a) and normal lung of control pigs (b); Typical interstitial pneumonia of HNjz15-infected pigs (c) and normal lung of control pigs (d); PRRSV-positive staining in the lungs of HNjz15-infected pigs (e) and control pigs (f).
was the original virus. At 14 dpi, all pigs were humanly euthanized and lung samples were collected for histopathology and immunochemistry examinations. The major findings of gross pathology were oedema and severe haemorrhage in lung (Fig. 4) and enlarged and swollen lymph nodes of HNjz15-infected pigs. Histopathological lesions were mainly found in lung of HNjz15-infected pigs. Interstitial pneumonia was characterized by thickening of alveolar septa and infiltration of mononuclear cells (Fig. 3). Lymph nodes exhibited characteristic lymphocyte depletion and haemorrhage. By contrast, no pathological lesions were observed in above organs in control pigs. Immunohistochemistry staining was used to reveal the presence of virus antigen. Immunohistochemistry experiments were conducted on paraffin-embedded sections using a specific anti-PRRSV N protein monoclonal antibody as previously described (Tian et al., 2007). Positive staining was detected in the lung and lymph nodes of pigs in HNjz15-infected group, and no positive staining was visible in above organs of pigs in control group. Therefore, all above data proved the clinical symptoms of infected pigs were caused by HNjz15 PRRSV infection.

Several studies reported the outbreaks of NADC30-like PRRSV in vaccinated pig herd with 30–50% fatality of pigs (Zhou et al., 2015a). In one study, one NADC30-like PRRSV strain JL580 showed high pathogenicity to 6-week-old pigs with mortality of 100% (Zhao et al., 2015). Genome-wide analysis of JL580 showed that it was a mosaic NADC30-like virus with HP-PRRSV 09HEN1 recombination at six different sites spanned the genome. We analysed the genome of HNjz15, and no recombination with other PRRSV strains was found (data not shown). Therefore, the incorporations of virulence-determining genes from HP-PRRSVs into the low pathogenic PRRSV such as NADC30 may lead to the change in virulence of mosaic recombinant virus, which may explain the discrepancy of pathogenicity between JL580 and HNjz15. However, this hypothesis needs to be proved by replacing these virulence-determining genes from HP-PRRSV to low pathogenic virus using reverse genetic techniques and vice versa. Besides virus recombination, other factors such as the virus titre for infection and proliferation ability of different virus strains may also account for the disparity of pathogenicity on pigs.

In conclusion, an epidemiology study was conducted to investigate the prevalence of NADC30-like PRRSV in south-east China. The survey results showed the disease already spread to several provinces in China. Although these NADC30-like PRRSVs showed different pathogenicities in experimental studies, the prevalence of these viruses in vaccinated pig herds proves the inefficacy of current commercial PRRSV vaccines and deserves more attention for PRRSV control in China.

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Conflict of Interest
None declared.

References
