RT-PCR detection of porcine reproductive and respiratory syndrome virus based on the ORF5 gene in mainland China, 2012–2015

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Summary. – Between January 2012 and December 2015, 13,567 clinical samples were collected from healthy pigs and pigs with a history of respiratory symptoms and/or reproductive disorders in 29 provinces of mainland China to detect porcine reproductive and respiratory syndrome virus (PRRSV). From these samples, 7490 were PRRSV-positive (average value, 55.21%). The annual PRRSV detection rate from 2012 to 2015 for each year is 45.67%, 55.99%, 56.91%, and 59.07%, respectively. Phylogenetic analyses revealed that the percentage of highly pathogenic PRRSV strains had a decreasing yearly trend, whereas the vaccine-like strains showed the opposite trend during the years 2012–2015. These data indicate that while the vaccine contributes to PRRSV prevention in China, it might also lead to serious problems. Specifically, we identified 12 NADC30-like PRRSVs during the years 2014–2015, suggesting potential transmission of the newly emerged PRRSV strains in China. Our data contribute to new information about the epidemiology of PRRSV.

Keywords: porcine reproductive and respiratory syndrome virus; recent profile; RT-PCR detection; ORF5 gene; China

Porcine reproductive and respiratory syndrome (PRRS) has been prevalent worldwide for almost 30 years since it was first reported in the USA in 1987. It has become one of the most economically significant diseases in the global pig farming industry (Han and Yoo, 2014). The causative agent of PRRS, the porcine reproductive and respiratory syndrome virus (PRRSV), is a single-stranded, positive-sense, enveloped RNA virus that belongs to the genus *Arterivirus*, the family *Arteriviridae* (Cavanagh, 1997). Two PRRSV genotypes have been defined based on the genomic heterogeneity as type 1, which comprises of the European type isolates represented by the Lelystad virus (LV), and type 2, which includes the North American (NA) type isolates represented by VR-2332 (Martínez-Lobo *et al*., 2011). These genotypes share only 55–70% nucleotide sequence identity (Forsberg *et al*., 2002; Han and Yoo, 2014). The PRRSV genome possesses ten open reading frames (ORFs). Of these, ORF1a and ORF1b encode pp1a and pp1ab, which are two non-structural polyproteins from which at least 16 non-structural proteins including nsp1α, nsp1β, nsp2, nsp2N, nsp2TF, nsp3–6, nsp7α, nsp7β, nsp8–12 are derived, while ORF2 to ORF7 encode the following eight structural proteins: GP2a, E (2b), GP3, GP4, 5a, GP5, M, and N (Chen *et al*., 2016). Among these ORFs, the ORF5 gene, which encodes the major envelope protein GP5, is one of the most variable regions in the PRRSV genome (Allende *et al*., 2000; Zhou *et al*., 2015), and is proposed therefore to be an optimal region for monitoring the evolution of PRRSV (Shi *et al*., 2010; Li *et al*., 2011; Xie *et al*., 2014).

Nine years after PRRS emerged in the USA, PRRSV was first reported in China in 1996, and the infections resulting from this *Arterivirus* have now become one of the gravest

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Abbreviations: LV = Lelystad virus; NA type = North America type; PRRS = porcine reproductive and respiratory syndrome; PRRSV = PRRS virus

threats to the country's pig herds, causing enormous economic losses each year (Xie *et al*., 2014). The 2006 atypical PRRS outbreak in China led to over 2,000,000 cases of infection, around 400,000 fatal cases, and more than 1,2 billion in Chinese Yuan in direct economic losses (Tian *et al*., 2007). Several variants of PRRSV strains such as JXA1, HEB1, HUB2 and HuN4 were proposed to be responsible for this outbreak (Tian *et al*., 2007; Tong *et al*., 2007). Similarly, the more recent widespread outbreaks of PPRS in several parts of China in 2014 are likely to be associated with the novel PRRSV NADC30-like strain, CHsx1401 (Zhou *et al*., 2015). Therefore, there is a pressing need to monitor the epidemic dynamics of this virus.

Between the years 2012 and 2015, a total of 13,567 clinical samples (lungs, kidneys, lymph nodes, tonsils and serum) comprising 2,492 samples in 2012, 3,424 samples in 2013, 3,915 samples in 2014, and 3736 samples in 2015 from pigs with a history of respiratory symptoms and/or reproductive disorders were collected from 29 provinces in mainland China, excluding Tibet and Ningxia (Fig. 1). The solid samples were homogenized using QIAGEN TissueLyser II (Qiagen, Germany) followed by total RNA extraction using an OMEGA Total RNA kit I (Omega, USA). Viral cDNA, used as the template in the RT-PCR (Li *et al*., 2015), was synthesized using MMLV reverse transcriptase (Takara, Japan). A pair of primers targeting the ORF5 gene (forward sequence from 5' to 3': GAG GTGGGCAACYGTTTTAG; reverse sequence from 5' to 3': CAMGMGTAGCGCCAGGACA) was designed and synthesized (BGI, China). Thermocycler conditions used for PCR were 5 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 58°C and 1 min at 72°C, and a final extension of 10 min at 72°C. The products were analysed on 1% gel electrophoresis. After that, the entire ORF5 sequences of all the PRRSV-positive samples were amplified using another pair of primers (F: GGCGACCGTTTTAGCCT GTCTT; R: ATCATTATTGGCGTGTAGGTG) using the same thermocycler conditions mentioned above and sequenced for further analysis. The products were firstly separated by electrophoresis on a 1% agarose gel, and then purified using a TIANgel midi purification kit (Tiangen, China) following the manual instructions and then cloned into a PMD18-T vector (Takara). Plasmids recovered from the positive colonies were further confirmed by DNA se-

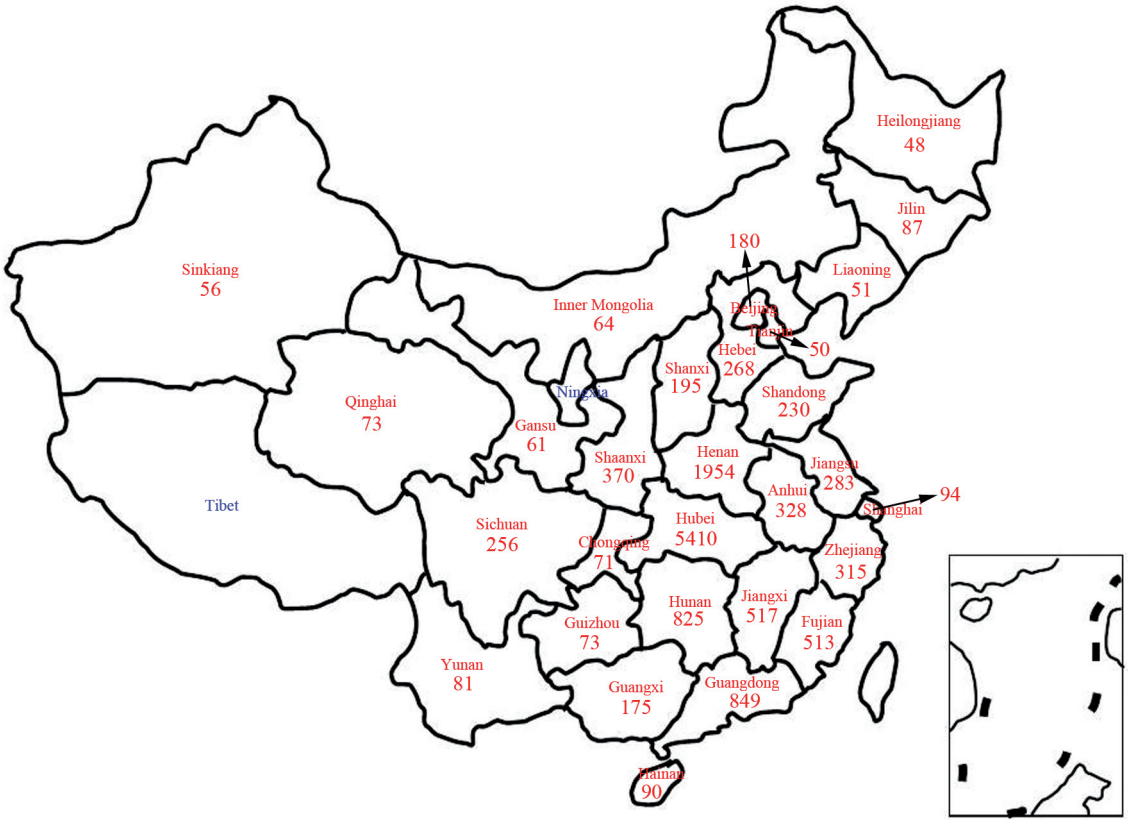

Fig. 1

Distribution of clinical samples collected from the 29 provinces in mainland China

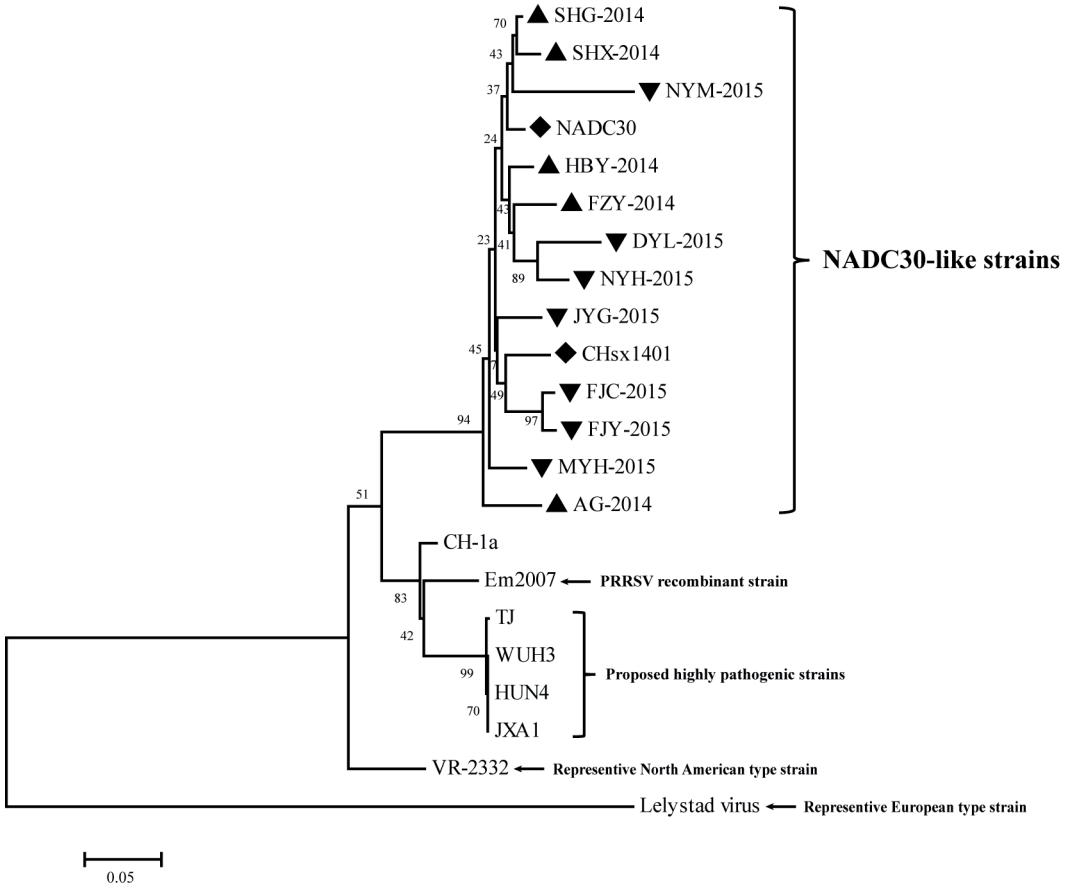

Fig. 2

Phylogenetic analyses of the ORF5 genes of the 12 PRRSV NADC30-like strains derived from this study

"▲" represents the NADC30-like strains determined in 2014 and "▼" indicates the NADC30-like strains identified in 2015. Representative prototype strains CHsx1401 (GenBank Acc. No. KP861625), TJ (EU860248), WHU3 (HM853673), HUN4 (EF635006), JAX1 (EF112445), Em2007 (EU262603), and CH1a (AY032626) were isolated from China. Of which PRRSV strain CHsx1401 is the novel NADC30-like strain reported in China in 2015 (Zhou *et al*., 2015); while Em2007 is reported as a PRRSV recombinant strain between PRRSV vaccine strains and PRRSV circulating strains (Li *et al*., 2009a). Representative prototype strains NADC30 (JN654459) and VR2332 (AY150564) were isolated from the United States. Prototype Lelystad virus (M96262) was used as the out group. The phylogenetic tree was constructed by using the distance-based neighbor-joining method with 1,000 bootstrap replicates in MEGA6. Numbers along branches are bootstrap values. Scale bar indicates nucleotide substitutions per site.

quencing (BGI). The assembled sequence data, as well as the publicly available ORF5 sequences of PRRSV strains from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) were used for the phylogenetic analysis. Multiple sequence alignments were performed using ClustalW (Thompson *et al*., 2002). A phylogenetic tree was generated by MEGA 6.0 software (Tamura *et al*., 2013) using the neighbor-joining algorithm with 1,000 bootstraps based on the comparative results exported from ClustalW.

From these samples, approximately 7,490 of the total samples investigated here were positive for PRRSV, as based on detection of the ORF5 gene, with an average positive rate of 55.21% (7,490/13,567). Annually, the PRRSV detection rate over the study period was 45.67% (1138/2492, 2012), 55.99% (1917/3424, 2013), 56.91% (2228/3915, 2014), and 59.07% (2207/3736, 2015), respectively, with an increasing yearly trend. Monthly, the highest positivity rates occurred in January, February, March and November. Seasonally, winter (December, January and February) is the season with a highest PRRSV detection rate over the past four years, while the highest values for the other seasons, in descending order, were autumn (September, October and November), summer (June, July and August) and spring (March, April and May) (Supplementary Table S1). For geographical distribution, the PRRSV detection rates in Northeast China, East China and South China were higher than for the other parts of China during the past four years (Supplementary Table S2).

The entire ORF5 sequences of the 7,490 PRRSV-positive samples were amplified and sequenced for phylogenetic analysis, which showed that all of the 7,490 strains were closely related to the NA type representative VR-2332 but far from the European type representative LV, suggesting that the current epidemic PRRSV in China is mainly the NA type virus. This finding is consistent with Li *et al.* (2011) who performed an epidemiological investigation of PRRSV in China between 2006 and 2010. Among the 7,490 strains, 27.36% of them was found to have close relationships with the ORF5 genes of the highly pathogenic PRRSV strains described previously (e.g., PRRSV strains HuN4 (Tong *et al.*, 2007), TJ (Leng *et al.*, 2008), JXA1 (Tian *et al.*, 2007), HEB1 (Tian *et al.*, 2007) and WuH3 (Li *et al.*, 2009b)), while 56.08% were close to the PRRSV vaccine strains (e.g., TJM (Leng *et al.*, 2012), JXA1-R (Leng *et al.*, 2012), HuN4-F112 (Tian *et al.*, 2009)). However, only 3.60% of the PRRSVs were similar to the classical NA PRRSV-type strains. The results suggest that the PRRSVs circulating in China in recent years are mainly the highly pathogenic-like strains. However, the highly pathogenic-like PRRSV positivity rate displayed a yearly decreasing trend (64.52% in 2012, 22.17% in 2013, 17.78% in 2014, and 20.00% in 2015). In comparison, detection of the vaccine-like strain increased annually (24.73% in 2012, 65.65% in 2013, 64.07% in 2014, and 59.53% in 2015). These findings indicate that wide use of PRRSV vaccines in China has helped to prevent PRRS, but the large scale use of these vaccines is becoming a serious problem, as was also revealed by the year-to-year percentage increase in the numbers of PRRSV recombinants between the vaccine strains and the circulating viruses. Indeed, the annual recombinant PRRSV strain prevalence from 2012 to 2015 was 16.74%, 20.70%, 24.30%, and 38.35%, respectively. In particular, 12 PRRSVs (5 obtained from 2014 and 7 obtained from 2015) are located in the same branch as the novel PRRSV NADC30 strain (Fig. 2). The PRRSV NADC30 strain, which was isolated in the USA in 2008, is defined as a highly pathogenic strain that can quickly induce viremia in infection-challenged pigs compared with other PRRSV isolates (Brockmeier *et al.*, 2012). The NADC30-like PRRSV in China, which was first reported in 2015, was proposed to be associated with severe outbreaks of PRRS in some intensive pig farms in China from August to December 2014 (Zhou *et al.*, 2015). The finding that 12 PRRSVs are genetically close to NADC30 PRRSV suggests the future potential for higher prevalence in the newly emerged NADC30-like PRRSV strains found in Chinese pig herds.

To conclude, the present study briefly reports the situation for PRRSV detection in China in the past four years (2012–2015). From these data we deduce that even the PRRSV strains circulating in Chinese pig herds in recent years are mainly the highly pathogenic-like strains, yet the prevalence of PRRSV has been restrained effectively, and this might be largely related to the wide use of PRRSV vaccines. However, relying on vaccines to prevent the spread of PRRSV and outbreaks of PRRS may also lead to serious problems. Attention should be paid especially to the potential prevalence of the newly emerged NADC30-like PRRSV strains in Chinese pigs, which may increase the difficulty of controlling PRRSV. The data retrieved from the present study will contribute to better understanding of the current prevalence of PRRSV in China.

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Supplementary information is available in the online version of the paper.

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Supplementary information

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Table S1. Monthly PRRSV positive rate of detection between 2012 and 2015

Season	Month	No. of total	No. of positive	Positive rate
	December	1317	744	56.49%
Winter	January	904	579	64.05%
	February	682	410	60.12%
Three months total (winter)		2903	1733	59.70%
	March	1586	989	62.36%
Spring	April	1249	671	53.72%
	May	1164	511	43.90%
Three months total (spring)		3999	2171	54.29%
	June	4681	2581	55.14%
Summer	July	1192	645	54.11%
	August	1111	575	51.76%
Three months total (summer)		6984	3801	54.42%
	September	1250	657	52.56%
Autumn	October	1095	601	54.89%
	November	1296	848	65.43%
Three months total (autumn)		3641	2106	57.84%

Table S2. Geographical PRRSV positive rate of detection between 2012 and 2015

Regions		Northeast	North China	East China	South China	Central China	Southwest	Northwest	Total
2012	No. of total	42	37	410	135	1645	172	51	2492
	No. of positive	29	31	231	95	686	42	24	1138
	Positive rate	69.05%	83.78%	56.34%	70.37%	41.70%	24.42%	47.06%	45.67%
2013	No. of total	162	224	376	184	2211	54	213	3424
	No. of positive	108	109	260	143	1201	39	57	1917
	Positive rate	66.67%	48.66%	69.15%	77.72%	54.32%	72.22%	26.76%	55.99%
2014	No. of total	24	249	866	324	2318	46	88	3915
	No. of positive	13	123	594	235	1198	30	35	2228
	Positive rate	54.17%	49.40%	68.59%	72.53%	51.68%	65.22%	39.77%	56.91%
2015	No. of total	18	161	772	468	2015	97	205	3736
	No. of positive	11	95	458	241	1191	65	146	2207
	Positive rate	61.11%	59.01%	59.33%	51.50%	59.11%	67.01%	71.22%	59.07%
Three years total	No. of total	246	671	2424	1111	8189	369	557	13567
	No. of positive	161	358	1543	714	4276	176	262	7490
	Positive rate	65.45%	53.35%	63.66%	64.27%	52.22%	47.70%	47.04%	55.21%