Acta virologica **62**: 229 – 234, 2018 doi:10.4149/av_2018_216

Diversity of group A rotavirus of porcine rotavirus in Shandong province China

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Received August 21, 2017; revised January 3, 2018; accepted June 29, 2018

Summary. – Porcine rotavirus (PoRV) is one of the major causes of neonatal diarrhea in swine worldwide. Multiple serotypes of PoRV have been detected in diarrhea cases of suckling and weaning pigs. To date, the prevalence and molecular characterizations of PoRV circulating in swine in Shandong province of China remains largely unknown. Two hundred and twenty-six feces samples were collected from ten farms showing diarrhea in Shandong. All the samples were tested by RT-PCR for the presence of PoRV, TGEV, or PEDV. The results showed that all farms are positive for PEDV, and 60% and 10% of the farms are positive for PoRV and TGEV respectively. PoRV was detected in 65 out of 226 (28.76%) samples collected from 1–3 months old suckling and weaning pigs, while the positive rates of the TGEV and PEDV were 2.21% and 34.96%, respectively. The present data emphasized that PoRV is an important pathogen causing diarrhea in swine in China. In addition, VP6 and VP7 genes of PoRVs were sequenced and analyzed. Phylogenetical analysis of VP6 showed that all of the five PoRVs belong to group A rotavirus, meanwhile VP7 genes belong to the G3, G5, and G9 genotypes. Moreover, G5 and G9 genotypes are the dominant genotypes. Taken together, co-infections of TGEV, PEDV, and PoRV occur in pig population in Shandong, and the multiple serotypes of PoRVs are circulating in those herds, suggesting the active surveillance and matched vaccine application.

Keywords: rotavirus; diarrhea; piglet; VP6; VP7; Shandong

Introduction

Diarrhea of piglets, induced by bacteria, viruses, or parasites, is a severe infectious syndrome, which often occurs in winter and spring and causes serious economic losses. Rotaviruses are an important etiologic agent of severe diarrhea among infants and young children, as well as the young of many species of animals. For instance, the bovine rotavirus was isolated from cattle worldwide (Hayashi-Miyamoto *et al.*, 2017; Papp *et al.*, 2013). The human rotavirus was found in humans by Bishop *et al.* (1979), and in the next year the porcine rotavirus was isolated from infected pigs by Woode *et al.* (1976). The piglets infected with porcine rotavirus (PoRV) show anorexia, restlessness, and occasional vomiting after the incubation period of 12–14 h. Even more, piglets suffers from high-capacity watery diarrhea, which not only leads to slow-growth in piglets, but also causes high mortality (Vlasova *et al.*, 2017).

Rotaviruses, members of the family *Reoviridae*, are non-enveloped icosahedral particles. Rotaviruses have been classified into eight groups (A-H) and two subgroups (I and II) based on the antigenic determinants of VP6 gene (Matthijnssens *et al.*, 2012). Among them, strains in group A, B, C, and H could infect humans and many other animal species (Wakuda *et al.*, 2011); nevertheless, strains in group D, E, F, and G have only been found in birds and pigs (Johne *et al.*, 2011). Five PoRV groups (RVA, RVB, RVC, RVE, and RVH) have been detected in pigs (Wakuda *et al.*, 2011), among these groups, group A rotavirus (RVA) is considered the

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Abbreviations: PoRV = porcine rotavirus; PEDV = porcine epidemic diarrhea virus; RVA = group A rotavirus; TGEV = transmissible gastroenteritis virus

most important in intestinal disease. RVA in piglets causes economic loses to swine industry worldwide. In UK, RVA positive samples rate reached 80% in fecal samples in 2009 and 2010 (Chandler-Bostock *et al.*, 2015). Molinari *et al.* (2016) reported that 61% of tested pig farms with diarrhea symptom were RVA and/or RVC positive in Belgium (Theuns *et al.*, 2016). In Brazil, 46% diarrhea samples from pigs were RVA positive (Molinari *et al.*, 2016).

The genome of rotaviruses is composed of 11 segments of double-stranded RNA encoding six structural proteins (VP1-VP4, VP6, and VP7) and five non-structural proteins (NS53, NS35, NS34, NS28, and NS26). VP6 gene encodes intermediate capsid protein which plays an important role in virus replication. Meanwhile, VP6 protein is the main determinant of species specificity. VP7 gene encodes outer-capsid glycoprotein (Mukherjee *et al.*, 2009). It is the main immunogen that induces neutralizing antibodies (Santos *et al.*, 2005). Rotavirus genotypes are very diverse, and among which, VP7 gene determines G-genotype (Estes and Cohen, 1989). Rotavirus strains within one G-genotype shares at least 90–91% VP7 amino acid sequence identity (Ciarlet *et al.*, 2002). To date, there is 27 G-genotypes that have been identified from many species (Matthijnssens *et al.*, 2011; Trojnar *et al.*, 2013). Until now, 12 G-genotypes (G1 to G6, G8 to G12, and G26) have been found in symptomatic and asymptomatic infections (Martella *et al.*, 2010; Papp *et al.*, 2013).

Shandong Province has the biggest swine industry in China, however, the prevalence and molecular characterization of PoRV circulating in swine in Shandong remains largely unknown.

Materials and Methods

Sample collection. Piglet diarrhea feces samples were collected from October 2013 to October 2014 from ten pig farms with diarrhea symptoms located in different areas in Shandong. A total of 226 piglet fecal samples were collected from diseased pigs in ten farms located in different cities in Shandong Province (Table 1)

Table 1. Detection rates of PoRV, PEDV, and TGEV in fresh feces collected from suckling and weaning pigs from ten farms located in different cities of Shandong province

Farms	Total samples	Number of positive sample			PEDV and PoRV co-infection	Number of pigs	Age
		TGEV	PEDV	PoRV			
Dezhou	31	0	5	5	2	800	3–4 weeks
Jinan	38	5	8	9	0	1000	4 weeks
Jining	33	0	7	10	2	600	5–6 weeks
Linyi	17	0	10	5	1	500	3 weeks
Taian	36	0	12	23	1	600	4 weeks
Binzhou	10	0	3	0	0	200	7 weeks
Weifang	48	0	27	13	1	1300	5–7 weeks
Rizhao	5	0	2	0	0	400	5 weeks
Dongying	5	0	3	0	0	300	3–5 weeks
Liaocheng	3	0	2	0	0	500	5 weeks
Total	226	5	79	65	7	6200	
Positive rate (%)		2.21%	34.96%	28.76%	3.10%		

Table 2. Sequence of primers designed to detect PEDV, TGEV, and PoRV

Primer name	Sequence (5'→3')	Size (bp)	Targeting gene
PEDV-F	CCGAGTGCGGTTCTCACAGA	1370	N
PEDV-R	CATAGCCAGGATAAGCCGGT		
TGEV-F	AGAACGCTATTGTGCTAT	697	M
TGEV-R	TCTTGCCTCTGTTGAGTA		
PoRV-F	GGCTTTTAAACGAAGTCTTC	1062	VP6
PoRV-R	GGTCACATCCTCTCACTA		
PoRV-VP4F	TCCATGGCTTCGCTCATTTATAGACA	876	VP4
PoRV-VP4R	GAGTAACCTCGAGGACCATTTATAACCCAAT		
PoRV-VP7F	GGCTTTAAAAGAGAGAATTTC	1026	VP7
PoRV-VP7R	GGTCACATCATACAGTTCTAAC		

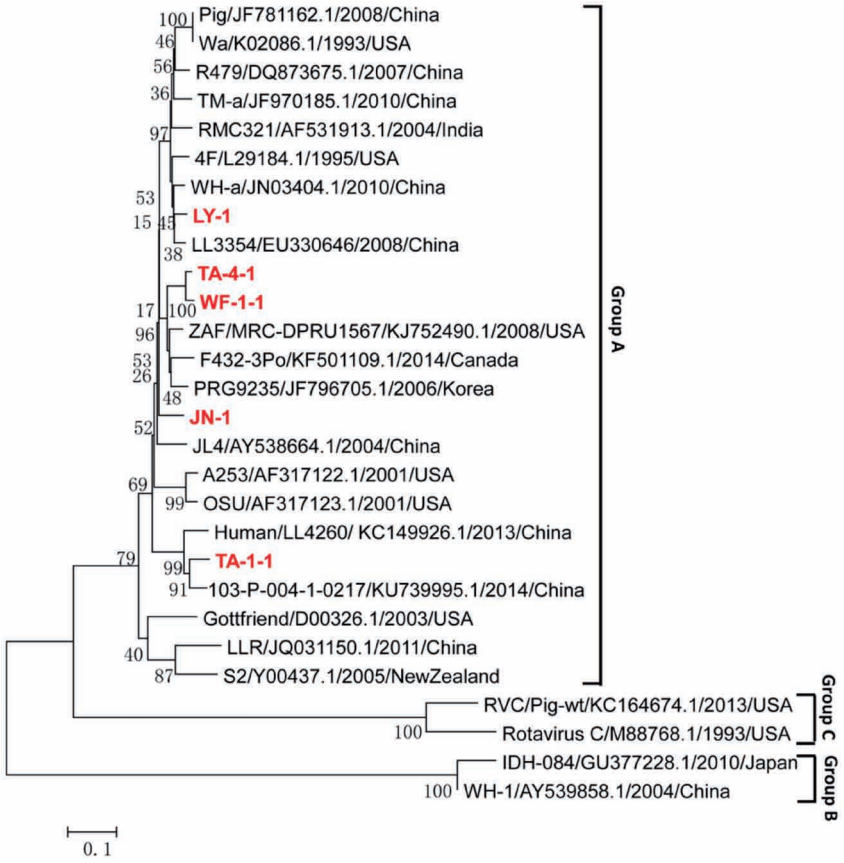

Fig. 1

Phylogenetic tree based on the nucleotide sequences of VP6 genes from different porcine rotavirus strains

The viruses isolated in this study are in bold and red. The names of strains are given: strain name/accession number/year/country. The scale represents genetic distance.

from October 2013 to October 2014. The animals were up to three weeks old and showed diarrhea of various severity. The samples were diluted in ratio of 1:5 with phosphate-buffered saline (PBS), and centrifuged at 5,000 x g for 10 min. The supernatants were collected for further analysis.

RNA isolation and RT-PCR. Viral RNA was extracted from fecal specimens using TRIzol® reagent according to manufacturer's instructions (Thermo Fisher, USA). One-step RT-PCR (Takara, Japan) was conducted in reaction mixtures consisting of 1 μl RNA, 2 μl of Prime Script® One Step enzyme mix, 1 μl of forward and reverse primer (20 μmol/l), 12.5 μl of 2× one-step buffer, and nuclease free water in a total volume of 25 μl per reaction. The RT-PCR conditions involved an initial reverse transcription of 30 min at 50°C, followed by PCR activation at 95°C for 10 min, 32 cycles of amplification (1 min at 94°C, 30 s at 53°C and 30 s at 72°C) with final extension at 72°C for 10 min. All RT-PCR products were analyzed on 0.8% agarose gel containing 0.5 μg/ml ethidium bromide and visualized under UV transilluminator.

Primers. To detect the three pathogens, three pairs of specific primers were designed to detect porcine epidemic diarrhea virus (PEDV) targeting N gene, transmissible gastroenteritis virus (TGEV) targeting M gene, and PoRV targeting VP6 gene from the samples. Another two pairs of primers targeting VP7 and VP4 genes of PoRV were designed and were used to amplify the full-length gene segments for further analysis. All primer sequences are summarized in Table 2.

Genotyping. To identify the genotypes of swine PoRV strains, the VP4, VP6, and VP7 were amplified and the PCR products were purified and cloned into pMD®18-T (Takara, Japan). VP4, VP6, and VP7 genes were sequenced by sequencing center in Shandong Academy of Agriculture Science. The sequences were assembled and edited using MEGA 6 software (Drummond and Rambaut, 2007). The nucleotide sequences derived in the present study were deposited in GenBank (KT820766 to KT820783). Molecular analysis was conducted by comparing the nucleotide sequences of VP4, VP6, and VP7 genes with those of reference strains available from GenBank. Alignments of multiple sequences were performed using the CLUSTAL W by MEGA 6 software. Phylogenetic trees were constructed via neighbor-joining method with 1,000 bootstrap replications.

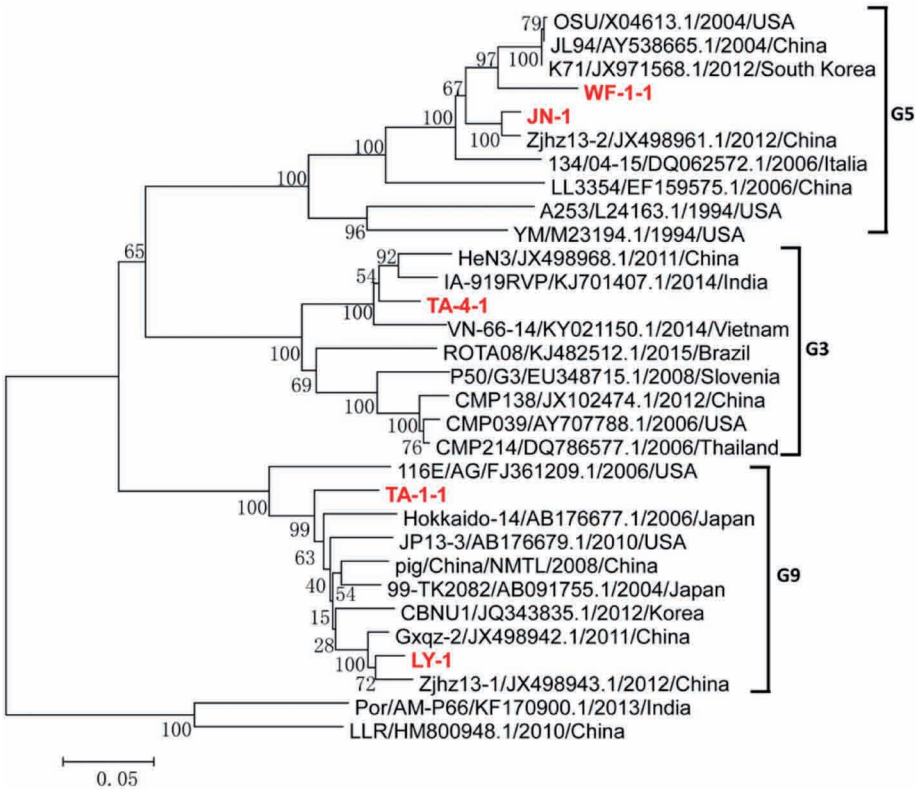

Fig. 2

Phylogenetic tree based on the nucleotide sequences of VP7 genes from different porcine rotavirus strains
The viruses isolated in this study are in bold and red. The names of strains are given: strain name/accession number/year/country. The scale represents genetic distance.

Results

Surveillance

The surveillance results showed that PoRV was identified in 65 out of 226 (28.76%) samples collected from 1–3 months old suckling and weaning pigs. The positive rates of TGEV and PEDV were 2.21% (5/226) and 34.95% (79/226), respectively. Furthermore, all investigated farms were positive for PEDV, and 60% and 10% of the farms were positive for PoRV and TGEV, respectively. Moreover, the positive rate of PoRV of one farm (Taian) was as high as 63.38% (Table 1). Our data for the first time indicated that PoRV tends to be the second most important pathogen causing diarrhea in swine in Shandong besides PEDV. In addition, 3.10% of the samples were positive for both PEDV and PoRV, suggesting the co-infection of these two pathogens among the pigs. However, the positive rate of TGEV was lower than the other two pathogens.

The presence of PoRV RNA in five positive samples (termed as LY-1, TA-1-1, TA-4-1, WF-1-1, JN-1) was confirmed by sequencing of VP4, VP6, and VP7 genes. The nucleotide sequences derived in the present study were deposited in GenBank with Acc. Nos. of KT820766 to KT820783.

Genotyping

The VP6 deduced amino acid sequence homologies among five rotavirus isolates ranged from 91.20% to 99.0%, and all isolates shared high similarities of 87.40% to 99.50% with the PoRV from group A, and lower identities with group C (35.0% to 41.20%) and group B (10.20% to 11.0%). Similarly, phylogenetic analysis showed that five rotavirus isolates were located in group A, suggesting that all the isolates belong to PoRV group A (Fig. 1).

Based on phylogenetic analysis and alignment of the five VP7 gene sequences with representative strains, the VP7 deduced amino acid sequence identities among five rotavirus isolates ranged from 81.3% to 99.7%. WF-1-1 and JN-1 strains showed high range of similarities (93.90% to 99.10%) with G5 representative strains (Fig. 2). LY-1 and TA-1-1 strains were clustered together with G9 reference strains (94.5% to 98.5%). It is interesting to point out that TA-4-1 strain, which was isolated from the same region as TA-1-1, was more closely related to G3 representative strains (93.60%) than to G9 strains (86.50% to 87.50%) (Fig. 2).

Based on phylogenetic analysis and alignment of VP4 gene sequences with representative strains, the VP4 de-

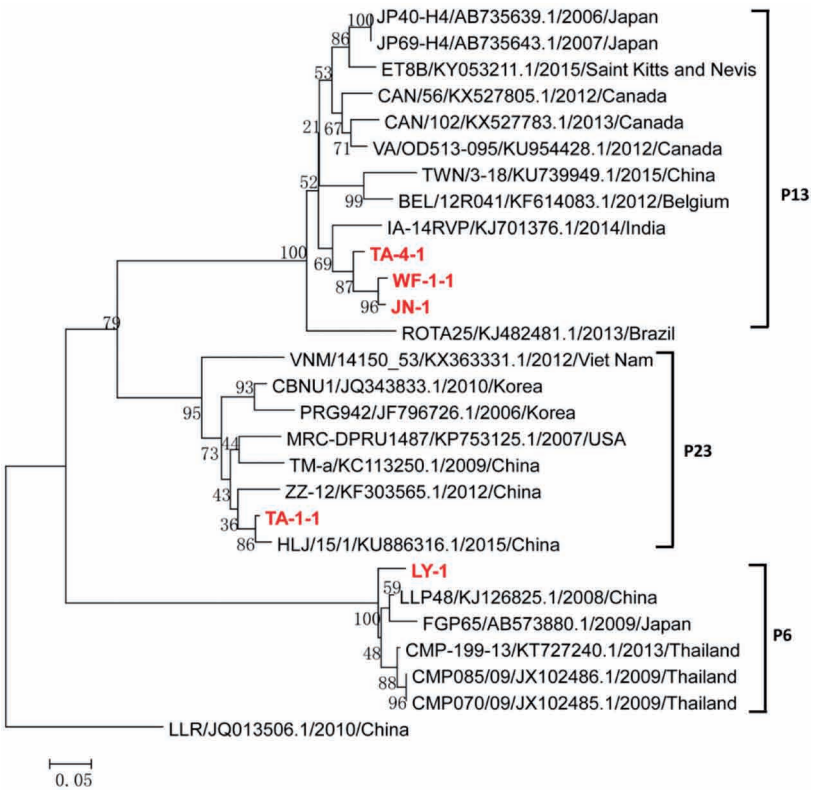

Fig. 3

Phylogenetic tree based on the nucleotide sequences of VP4 genes from different porcine rotavirus strains

The viruses isolated in this study are in bold and red. The names of strains are given: strain name/accession number/year/country. The scale represents genetic distance.

duced amino acid sequence identities among five rotavirus isolates ranged from 81.3% to 99.7%. Phylogenetic trees indicated that TA-4-1, WF-1-1, and JN-1 strains showed close relationship with P13 representative strains (Fig. 3). TA-1-1 strains were clustered together with P23 reference strain, and LY-1 was more closely related to P6 representative strain (Fig. 3).

Discussion

PoRV is one of the main pathogens of porcine diarrhea, however, it is difficult to differentiate it from PEDV and TGEV only by clinical signs. PoRV not only causes diarrhea, but also inhibits the immune system, resulting in growth arrest and mortality increase in piglets. In this study, 100% of the investigated farms are positive for PEDV, 10% and 60% of the farms are positive for TGEV and PoRV, respectively. Additionally, 28.76% and 34.95% of the collected samples are positive for PoRV and PEDV, respectively. PEDV is a major pathogen causing diarrhea in swine in China, however, the circulation situation of PoRV remains largely unclear. Our data are the first to indicate that PoRV seems to be the second important pathogen causing diarrhea in swine in China.

VP6 is an important group antigen of PoRV, associated with VP4 and VP7 to constitute the framework of the virus. Through phylogenetic analysis, all of the five isolated viruses were located in the same branch with group A viruses. The results showed that, during the year of 2013 and 2014, group A rotaviruses were the epidemic group circulating in pig herds in Shandong Province.

VP7 gene is the main immune protective antigen, which not only stimulates the body to produce antibodies but also determines the G-genotype of PoRV (Kim *et al.*, 2013). Previous studies suggest that highly variable area is located in VP7 genetic region (Coulson and Kirkwood, 1991). The antigenic site encoded by VP7 gene has little cross protection among different genotypes (Diwakarla and Palombo, 1999).

This study revealed that multiple genotypes of PoRV are circulating in Shandong, China. For instance, the WF-5-2 strain isolated from Weifang region belongs to G5 genotype, but LY-2 strain belongs to G9 genotype which was isolated from Linyi region. Interestingly, the PoRV isolated from the Taian region belongs to different genotype, such as the TA-1-2 and TA-4-2 strains isolated from the same place of

Taian region belonging to genotype G9 and G3. Therefore, vaccines should be used rationally depending on different genotypes circulating in different regions.

Acknowledgments. This study was supported by the National Natural Science Foundation of China (No. 31772753, No. 31572543, No. 31472206 and No. 31402150), and Chinese Academy of Agricultural Sciences Young Talent Scientist Program (No. CAASQNY-CKYYJ58), Epidemiological Survey of Diarrhea Piglet and Study of Synthesize Control of Shandong Province (No. 2013GC15003).

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