Identification and characterization of porcine parvovirus in Hunan province, China¹⁾

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Received 18.02.2020

Accepted 19.04.2020

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Summary

Porcine parvovirus (PPV) is considered one of the most important infectious agents of reproductive failure in sows. Little information, however, is available on its prevalence in healthy fattening pigs. Therefore, in the present study, 197 fecal swabs, 197 nasal swabs, 389 serum samples, and 310 lung samples were collected from pigs aged 10-25 weeks across Hunan, China, and tested for the presence of PPV. PPV DNA was amplified by polymerase chain reaction, demonstrating an overall positivity rate of 7.69%, with a particularly high infection rate of 22.90% in the lungs. A total of five PPV strains (PPV-HuN1-5) were isolated on the basis of cytopathic effects in swine testicular cells, and the near-complete genomes were sequenced and subjected to phylogenetic analysis with reference to reported PPV sequences in GenBank. The five Hunan isolates showed a close relationship with each other and predominantly with reported European PPV strains. Moreover, seven amino acid substitutions were detected within the coding region of the VP2 of PPV-HuNs when compared with that of the Chinese vaccine strain PPV-NJ. The relatively high prevalence of PPV discovered in healthy fattening pigs despite a long-term vaccination program in China, highlights the need for improved prevention, monitoring, and control of related diseases in herds.

Keywords: porcine parvovirus, prevalence, fattening pigs, phylogeny

Taxonomically, *Ungulate protoparvovirus 1*, also known as *Porcine parvovirus* (PPV), belongs to the genus *Protoparvovirus* of the subfamily *Parvovirinae* within the family *Parvoviridae* (5). The PPV genome consists of a negative-sense single-stranded DNA molecule of about 5 kb in size. Both terminals of the virus genome form complex palindromic hairpin structures in a Y- or T-like shape (22). The PPV genome comprises two major open reading frames (ORFs), encoding seven proteins by the complementary strand: ORF1 and ORF2 encode non-structural proteins (NS1, NS2, and NS3) and structural proteins (VP1, VP2, and VP3), respectively (20). Furthermore, a late non-structural protein (SAT) is expressed from the same VP2 mRNA, initiated at the seven nucleotides downstream of the VP2 start codon (25).

PPV was first isolated in Germany in 1965 as a contaminant of porcine primary cell cultures (13). Since then, PPV has been identified worldwide, including in Africa, Asia, Australia, Europe, and North America (1, 4, 6, 17, 19). In most cases, PPV infection alone

does not cause clinical symptoms in non-pregnant adult pigs or piglets. However, virulent strains can cause reproductive failure in swine herds, and can lead to clinical still birth, mummification, embryonic death, and infertility (SMEDI) syndrome (14). Despite the wide use of PPV vaccines in China, PPV has remained a constant problem in the pig industry, and several studies reported continuous outbreaks of PPV in pig populations (8, 19). However, the general prevalence of PPV is still not well understood in most parts of China. Therefore, the present study was conducted to characterize PPV and to determine its overall prevalence in apparently healthy domestic pigs throughout the Hunan province, China.

Material and methods

Sample collection. A total of 1093 samples were collected from pig farms and slaughterhouses across the Hunan province from January to August 2017, including 197 fecal swabs, 197 nasal swabs, 389 serum samples, and 310 lung samples. To avoid possible cross-contamination, the samples were collected with sterile instruments and on different days whenever possible. The fecal swabs, nasal swabs, and serum samples were obtained from nine pig farms (Tab. 1),

¹⁾ This work was supported by the National Key Research and Development Program of China (2017YFD0500104).

Tab. 1. Detailed information on samples and prevalence of PPV in different farms

Farm	No. of samples	Fecal swabs	Nasal swabs	Serum
		PPV-positivity rate (%)		
1	87	3.48 (1/29)	6.90 (2/29)	6.90 (2/29)
2	144	2.08 (1/48)	0 (0/48)	0 (0/48)
3	72	4.17 (1/24)	4.17 (1/24)	0 (0/24)
4	144	4.17 (2/48)	2.08 (1/48)	0 (0/48)
5	144	0 (0/48)	0 (0/48)	0 (0/48)
6	48	–	–	0 (0/48)
7	32	–	–	0 (0/32)
8	48	–	–	0 (0/48)
9	64	–	–	3.13 (2/64)
Overall	783	2.54 (5/197)	2.03 (4/197)	1.03 (4/389)

Explanation: – Not identified

Tab. 2. Primers for PPV detection and ORFs gene amplification

Primers	Oligonucleotide sequence (5'-3')	Position ^a	Annealing temperature/°C
PPV-F1 ^b	ACTCTCAGCTACTGCAGCAT	273	55
PPV-R1 ^c	TGCATTATTAACCATCTACTCCAT	775	
PPV-F2	GTGGAAAAGGCTTACAACAA	692	
PPV-R2	GGTTTTGCCITTTCAAGTATTA	1337	55
PPV-F3	TAGAAATGATGGCTCAAACC	1220	
PPV-R3	GCTGCTGAGAAGTAGAAGTA	2544	
PPV-F4	AAAAGAGCAAGAGGTAAGGG	2302	55
PPV-R4	TGACCAAGGTGTACCATTT	3139	
PPV-F5	CGCATCAAGACTCATACATC	3004	
PPV-R5	GTCAGCATTGAAATCATCTGTTAG	4336	59
PPV-F6	ACAGCACTAAACAATACTGCACCT	4161	
PPV-R6	CTTGGTATAAGTTGTGAATATTCTG	4539	

Explanation: a – Location of primers relative to the complete genomic sequence of PPV/NADL-2 (GenBank # NC001718); b – F: forward primer; c – R: reverse primer

whereas the lung samples were collected from a slaughterhouse in the city of Changsha in the Hunan province. All of the samples came from healthy pigs without apparent clinical signs. The age of the pigs sampled ranged from 10 to 25 weeks. The samples were shipped on ice and kept in a freezer at –80°C until use.

Viral DNA extraction and polymerase chain reaction (PCR) for detecting PPV. After re-suspending the fecal and nasal swabs in 0.5 ml of sterile phosphate-buffered saline (PBS), they were centrifuged at $7,000 \times g$ for 5 min, and 0.2 ml of the suspension was transferred into a 2-ml tube that was used for viral DNA extraction. The serum samples were centrifuged at $5,000 \times g$ for 3 min, and 0.2 ml of the suspension was transferred and used for viral DNA extraction. For tissue samples, 0.5 g aliquots were placed into 2 ml Eppendorf tubes containing 1 ml of sterile PBS with four 1.5 mm steel balls, and then ground into powder with a Mixer Mill MM 400 (Retsch, Germany) for 3 min. The samples were then clarified by centrifugation at $12,000 \times g$ for 10 min. DNA was extracted according to the manufacturer's instructions (Tiandz Inc., Beijing, China), and the

Tab. 3. Porcine parvovirus reference strains used in the study

Virus isolate	GenBank accession No.	Geographic origin	Dataset	Collection date
32350005_3h	GQ884039	Europe	ORFs	2005
32260005serh_1h	GQ884036	Europe	ORFs	2005
1242/2012	MH558678	Spain	ORFs	2012
14a	JN400518	Germany	ORFs	2009
8a	JN400517	Germany	ORFs	2009
7a	JN400516	Germany	ORFs	2009
33800005_1g	GQ884044	Europe	ORFs	2006
JY	HM627652	China	ORFs	2010
32260005_1d	GQ884037	Europe	ORFs	2005
32340005_2f	GQ884038	Europe	ORFs	2005
33760005_3a	GQ884041	Europe	ORFs	2006
693a	JN400519	Austria	ORFs	2009
33780005_2g	GQ884042	Europe	ORFs	2006
Challenge	AY684866	United Kingdom	ORFs	1986
Kresse	U44978	USA	ORFs	1985
40570005_4e	GQ884047	USA	ORFs	2006
HN-2011	JX992846	China	ORFs	2008
ZJ	EU790642	China	ORFs	2004
HNLY201301	MF447833	China	ORFs	2013
NADL-2	L23427	USA	ORFs	1976
YL	JN860197	China	ORFs	–
T142_Korea	KY994646	South Korea	ORFs	2016
LZ	HM627653	China	ORFs	2009
BQ	EU790641	China	ORFs	2006
HN-H	KF429253	China	ORFs	2012
PPV2010	JN872448	China	ORFs	2010
SR-1	DQ675456	China	ORFs	–
GD2013	KX242359	China	ORFs	2013
HN-I	KF429254	China	ORFs	2013
HN-G	KF429252	China	ORFs	2012
JT_NS3	JN968975	China	ORFs	2010
33790005_6a	GQ884043	Europe	ORFs	2006
40090005_1h	GQ884045	USA	ORFs	2006
J-PPV	KF742500	China	ORFs	2013
HN-K	KF429255	China	ORFs	2013
21620005_1h	GQ884035	Europe	ORFs	2005
N	HM989009	China	ORFs	1989
China	AY583318	China	ORFs	2004
32360005_1f	GQ884040	Europe	ORFs	2005
VRI-1	AY390557	South Korea	ORFs	2003
PPV/Tornau/1/02	AY684869	Germany	ORFs	2002
VaccinevirusIDT	AY684872	Germany	ORFs	1964
N108	MH566237	South Korea	ORFs	2018
HNZK-1	KJ201928	China	ORFs	2007
HNAY	KJ201927	China	ORFs	2009
16WS	KM268633	China	ORFs	2013
TJ	KX233726	China	ORFs	2015
Nanjing200801	FJ822038	China	ORFs	2008
N91	MH817778	South Korea	ORFs	2018
N2	MH817779	South Korea	ORFs	2018
NJ	AY686601	China	NS1	–
NJ	AY686602	China	VP2	–

Explanation: – Not identified

Fig. 1. Alignment of the VP2 amino acid sequences of PPV-HuNs with the Chinese vaccine strain PPV-NJ. Sequences in the boxes show the amino acid substitutions.

Tab. 4. Homology comparison of porcine parvovirus complete ORFs genes

Strain ^a	% nucleotide identities:				
	HuN1	HuN2	HuN3	HuN4	HuN5
HuN1					
HuN2	100.0				
HuN3	99.9	99.9			
HuN4	99.9	99.9	99.8		
HuN5	99.9	99.9	99.9	99.8	
32350005_3h	99.9	99.9	99.9	99.8	99.9
32260005erh_1h	99.7	99.7	99.8	99.6	99.7
2012	99.7	99.7	99.7	99.6	99.7
14a	99.7	99.7	99.7	99.6	99.7
8a	99.6	99.6	99.6	99.5	99.6
7a	99.6	99.6	99.6	99.5	99.6
33800005_1g	99.6	99.6	99.6	99.5	99.6
JY	99.6	99.6	99.6	99.5	99.6
32260005_1d	99.4	99.4	99.5	99.3	99.4
2f	99.4	99.4	99.4	99.3	99.4
33760005_3a	99.3	99.3	99.3	99.2	99.3
693a	99.3	99.3	99.3	99.2	99.3
33780005_2g	99.2	99.2	99.2	99.1	99.2
Challenge	99.2	99.2	99.2	99.1	99.2
Kresse	99.2	99.2	99.2	99.1	99.2
40570005_4e	99.1	99.1	99.2	99.0	99.1
HN-2011	99.2	99.2	99.2	99.1	99.2
ZJ	99.2	99.2	99.2	99.1	99.2
HNLY201301	99.1	99.1	99.2	99.0	99.1
NADL-2	99.1	99.1	99.2	99.0	99.1
YL	99.1	99.1	99.1	99.0	99.1
T142_Korea	99.1	99.1	99.1	99.0	99.1
LZ	99.1	99.1	99.1	99.0	99.1
BQ	99.1	99.1	99.1	99.0	99.1
HN-H	99.1	99.1	99.1	99.0	99.1
PPV2010	99.1	99.1	99.1	99.0	99.1
SR-1	99.1	99.1	99.1	99.0	99.1
GD2013	99.0	99.0	99.1	98.9	99.0
HN-I	99.0	99.0	99.0	98.9	99.0
HN-G	99.0	99.0	99.0	98.9	99.0
JT_NS3	99.0	99.0	99.0	98.9	99.0
33790005_6a	99.0	99.0	99.0	98.9	99.0
40090005_1h	99.0	99.0	99.0	98.9	99.0
J-PPV	99.0	99.0	99.0	98.9	99.0
HN-K	99.0	99.0	99.0	98.9	99.0
21620005_1h	99.0	99.0	99.0	98.9	99.0
N	99.0	99.0	99.0	98.9	99.0
China	99.0	99.0	99.0	98.9	99.0
32360005_1f	98.9	98.9	99.0	98.9	98.9
VRI-1	99.0	99.0	99.0	98.9	99.0
2	98.9	98.9	98.9	98.8	98.9
VaccineVirusIDT	98.9	98.9	98.9	98.8	98.9
N108	98.8	98.8	98.8	98.7	98.8
HNZK-1	98.8	98.8	98.8	98.7	98.8
HNAY	98.8	98.8	98.8	98.7	98.8
16WS	98.8	98.8	98.8	98.7	98.8
TJ	98.7	98.7	98.8	98.6	98.7
Nanjing200801	98.7	98.7	98.7	98.6	98.7
N91	98.6	98.6	98.6	98.5	98.6
N2	98.4	98.4	98.4	98.3	98.4

Explanation: a – Details of reference strains in Supplementary Table 2.

Fig. 2. Phylogenetic tree based on the complete ORFs sequences of all available PPV strains. The Hasegawa-Kishino-Yano model with the proportion of invariant sites and gamma-distributed rate heterogeneity was used to construct the Maximum Likelihood tree with 1000 bootstrap replications to test the phylogeny. The percentage of replicates in which the associated virus clustered together in the bootstrap test (1000 replicates) is shown next to the branches in each tree (only values >70% are shown). The scale bar indicates nucleotide substitutions per site. The strains isolated in the present study are identified by ▲.

Homology comparison of the complete ORF sequences of PPV-HuN1-5 with all other reference strains in GenBank showed the closest relationship to the PPV-32350005_3h sequence (GQ884039) from Europe, with an average nucleotide homology of 99.9% (Tab. 4). Moreover, in the phylogenetic analysis of complete ORFs, the five PPV-HuNs clustered in the same clade and showed a close relationship to PPV strains from Europe (e.g., 32350005_3h, 32260005erh_1h, 33800005_1g, 1242/2012, and 14a) and China (JY) (Fig. 2).

Since PPV was first recognized at the end of the 1960s, it has been discovered in pigs worldwide, being one of the most common and important infectious agents of infertility and causing economic losses to the swine industry (15). PPV outbreaks have been reported in several countries (8, 11). In general, PPV infection during the first half of pregnancy can lead to the reproductive failure of sows, but does not cause clinical symptoms in non-pregnant adult pigs or piglets (15). According to studies on abortion cases with clinical and experimental infection of PPV field viruses, PPV antigens can be detected in multiple tissues, including the heart, liver, kidney, duodenum, thymus, lung, spleen, brain, and lymph nodes of fetuses, as well as in the placenta and uterus of sows (11, 24). However, information on the epidemiology and tissue tropism of PPV infection in healthy non-pregnant adult pigs is scarce. In the present study, we detected a high prevalence of PPV in the lungs of apparently healthy fattening pigs, whereas the detection rates in fecal swabs, nasal swabs, and serum samples were low. This finding may indicate tissue tropism to the lungs. However, further studies with more samples and sample types are needed to confirm these findings to clarify the tissue tropism and distribution of PPV in healthy non-pregnant adult pigs.

Although an inactivated PPV vaccine has been widely used in China, only sows are typically inoculated in most herds. In general, the maternal antibodies produced from the vaccine can offer passive protection to the piglets until 9-22 weeks of age (7). Therefore, the finding of this high prevalence of PPV in slaughtered pigs which were no longer passively protected from milk but not yet vaccinated was not unexpected. Moreover, the rare presence of PPV in fecal swabs, nasal swabs, and serum might indicate short-term shedding of the virus and viremia in healthy fattening pigs after PPV infection. Nevertheless, as fattening pigs represent the largest population in the herd, such long-term PPV carriers would pose a high risk of spread and a challenge for PPV disease prevention and control.

PPV was previously considered to have a conservative genome (12). However, recent studies based on systematic analyses of the genetic variation and evolution of the virus indicated high mutation rates (approximately 10^{-4}) in the VP genes, with a more moderate evolutionary rate for NS genes (approximately 10^{-5}) (18, 21). Although several studies focused on

the genetic diversity of PPV field strains from both domestic pigs and wild boars, the classification of PPV subgroups remains ambiguous (3, 16, 18). Moreover, no obvious correlation has been observed between clustering from phylogeny and geographical distribution or the virulence status of PPV isolates (15). The present findings fit with this general pattern, as the five PPV-HuN isolates obtained in this study from Hunan showed a relatively closer genetic relationship with each other, and phylogenetic analysis of complete ORFs demonstrated a close relationship of these isolates with most of the European PPV strains.

Inactivated vaccines have been widely used and have conferred good protection against PPV disease for a long time. However, recent studies conducted in Germany indicated that although these inactivated vaccines are effective against homologous infections, they could not prevent infection and virus shedding after challenge with the antigenically heterologous 27a strain (9, 26). The determinants of PPV virulence have not yet been identified, but several studies suggest that the amino acid residue substitutions in accessible regions on the capsid surface, especially the positions of linear epitopes on VP2, play an important role in the pathogenicity of PPV *in vivo* (10, 15, 22). Indeed, we identified seven amino acid substitutions in the coding region of VP2 between PPV-HuN1-5 and the Chinese vaccine strain PPV-NJ. Three of these amino acid positions (378, 383, and 436) are considered to be responsible for host tropism in tissue culture cells (2). A three-dimensional model of PPV VP2 predicted amino acids 378 and 383 to be located close to the 2-fold axis on the surface of the virus capsid, and position 436 is located within the 3-fold spike center of the capsid subunit, indicating that these sites may be involved in the immune response and virulence (22). However, further investigation is needed to clarify the precise influence of these amino acid variations on the capsid structure and immune response.

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