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The rate of co-infection for piglet diarrhea viruses in China and the genetic characterization of porcine epidemic diarrhea virus and porcine kobuvirus

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Summary. – Piglet diarrhea epidemics result in major economic losses for the swine industry. Four viruses are closely linked to porcine diarrhea: porcine kobuvirus (PKV), porcine epidemic diarrhea virus (PEDV), porcine transmissible gastroenteritis virus (TGEV), and porcine rotavirus (PRoV). We have conducted an epidemiology study to determine the frequency of infection and co-infection with these viruses in China, and characterized the genetic variation of the isolated PEDV and PKV strains. Stool and intestinal samples (n = 314) were collected from piglets with diarrhea in China from years 2012 to 2014. RT-PCR was used to detect PKV, PEDV, TGEV, and PRoV. Phylogenetic relationships between reference strains and the isolated PEDV and PKV strains were determined based on the M and 3D gene sequence. The rates of infection with PKV, PEDV, TGEV and PRoV were 29.9%, 24.2%, 1.91%, and 0.31%, respectively. Co-infections with PKV and the other three viruses were very common. Co-infection of PKV and PEDV was detected in 15.0% (47/314) of the samples. Phylogenetic analysis of the PKV 3D gene indicated that there were some phylogenetic differences in the PKV strains across regions within China. However, according to the PEDV M gene, strains clustered into three groups and the primary group was distinct from the vaccine strain CV777. This study provides insights in to the prevalence of diarrhea viruses and their prevention and control in China.

Keywords: porcine diarrhea viruses; co-infection; porcine kobuvirus; porcine epidemic diarrhea virus; porcine gastroenteritis; porcine rotavirus

Introduction

Viral diarrhea is a major cause of morbidity and mortality in piglets. The principal etiological agents of most viral diarrhea and malabsorption diseases in piglets are: transmissible gastroenteritis virus (TGEV), porcine epidemic diarrhea virus (PEDV), and porcine rotavirus (PRoV). PEDV and TGEV are enveloped, single-stranded RNA viruses belonging to the *Coronaviridae* family. PRoV is a double-stranded, non-enveloped RNA virus belonging to the *Reoviridae* family. All three viruses cause an acute and highly contagious enteric disease characterized by severe enteritis, watery diarrhea, vomiting, and dehydration that is associated with a high degree of mortality in suckling piglets (Coussement *et al.*, 1982; Laude *et al.*, 1990; Prabha and Verghese, 2009).

A fourth possible etiological agent of diarrhea in piglets is porcine kobuvirus (PKV), a member of the *Kobuvirus* genus in the *Picornaviridae* family. PKV is a non-enveloped, single-stranded, positive-sense RNA virus that was first detected in Hungary in early 2007 (Reuter *et al.*, 2008), and has now been detected worldwide (Khamrin *et al.*, 2010; Sisay *et al.*, 2013; Yu *et al.*, 2009). While the pathogenesis of PKV is relatively unknown, Park *et al.* suggested that PKV could be the etiological agent of gastroenteritis in pigs based on

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Abbreviations: PKV = porcine kobuvirus; PEDV = porcine epidemic diarrhea virus; TGEV = porcine transmissible gastroenteritis virus; ProV = porcine rotavirus

statistical analysis (Park *et al.*, 2010). Infections with PEDV, PKV, TGEV, and PRoV were all reported in an epidemic of porcine diarrhea in 2010 in China (Cao *et al.*, 2012; Shi *et al.*, 2013; Zhao *et al.*, 2013).

Co-infection with these viruses makes preventing and curing diarrhea in pigs more complex. Therefore, we performed an epidemiological study to identify the frequency of these viruses in cases of porcine viral diarrhea in China. We also characterized the genetic variation and molecular epidemiology of PEDV and PKV, which represent major potential threats. The membrane (M) protein of PEDV and 3D protein of PKV were conservative but important for viral replication, and they were therefore chosen for phylogenetic analysis.

Materials and Methods

Sample collection. Intestine or stool samples (n = 314) were collected from piglets with acute enteritis or watery diarrhea collected from throughout Jilin, Henan, Jiangsu, Shanghai, and Guangdong provinces between the years 2012 and 2014. The samples were diluted nine-times by phosphate-buffered saline (PBS, 0.1 M, pH 7.2), vortexed, and then centrifuged at 2,000 × g for 10 min at 4°C. The supernatants were then stored at -80°C until they were used for analysis. The experiments were performed according to the institutional animal care guidelines and approved by the Animal Care Committee of College of Veterinary Medicine, Yangzhou University.

RT-PCR. Total RNA was extracted from the stored sample supernatants using TRIzol (Invitrogen, USA) according to the manufacturer's instructions. Reverse transcription was performed using the SuperScript® III first-strand synthesis system (Invitrogen, USA), and then the synthesized cDNA was used for PCR. Primer sequences were designed to detect PKV, PEDV, TGEV, and PRoV based on the reference sequence for each virus (Table1). PCR reactions were carried out in a 25 µl volume in PCR buffer containing LA Taq polymerase (2.5 U; TaKaRa, China), $MgCl_2$ (1.5 mmol/l), template DNA (20 ng), the forward and reverse primers (10 µmol/l each), and dNTPs (2.5 µmol/l each). The fragments were amplified using the following reaction conditions: one cycle at 95°C for 5 min; followed by 35 cycles consisting of denaturation at 95°C for 1 min, annealing at 55°C (PEDV, TGEV), 52°C (PKV), or 53.5°C (PRoV) for 45 sec, and amplification at 72°C for 1 min; and a final extension step lasting 10 min at 72°C. PCR products were then visualized on a 1.2% agarose gel.

DNA cloning and sequence analysis. To investigate the molecular epidemiology and genetic variation of PEDV and PKV, the bands from 16 PEDV-positive samples and 15 PKV-positive samples (Table 2) were cloned and sequenced. The bands were excised from the 1.2% agarose gel, and then purified using the QIAquick gel extraction kit (QIAGEN, Germany) according to the manufacturer's instructions. The purified DNA was cloned into the pGEM®-T Easy vector (Promega, USA), and these constructs were used to transform *E. coli* DH5α competent cells. The resulting plasmids were sequenced by Invitrogen. Finally, the nucleotide sequences and deduced amino acid sequences were compared to other PEDV and kobuviruses in GenBank (Table 3). Sequence similarity analysis was performed using the ClustalW method using the Megalign 7.2 program. Phylogenetic analysis was carried out based on nucleotide alignments using the MEGA 5.0 software (Tamura *et al.*, 2011).

Results

Virus detection in samples collected from the field

Of the 314 samples collected in the field 94 (29.9%) were positive for PKV, 76 (24.2%) were positive for PEDV, 6 (1.91%) were positive for TGEV, and only 1 (0.31%) was positive for PRoV. Infection with PKV alone was detected in 45/314 samples (14.3%). The remaining PKV positive samples (49/94; 52.1%) were co-infected with PEDV (47/94; 50%), TGEV (1/94; 1.1%), or PRoV (1/94; 1.1%). Infection with only PEDV and TGEV was documented in 29/314 (9.2%) and 5/314 (1.6%) samples, respectively. The only case of PRoV was a co-infection with PKV. We did not observe

Table 1. Primer pairs used to detect porcine diarrhea viruses in stool and intestinal samples

Virus	Primer sequence	Product length	Target gene	Reference strain
PKV	F:5'-TGGATTACAAGTGTTTT-3'	217 bp	3D	S-1-HUN (NC011829)
	R:5'-ATGTTGTTAATGATGGT-3'			
PEDV	F:5'-GGACACATTCTTGGTGGT-3'	370 bp	M	CV777 (AF353511)
	R:5'-GTTTAGACTAAATGAAG-3'			
TGEV	F:5'-GATTTGATTTGGCAATGC-3'	102 bp	7b	WH-1 (HQ462571)
	R:5'-AACAATCACTAGATCCAG-3'			
PRoV	F:5'-TGGTATTGAATATACCAC-3'	802 bp	VP4	JL94 (AY523636)
	R:5'-CTGTTGGCCACCCTTTAG-3'			

PKV = porcine kobuvirus; PEDV = porcine epidemic diarrhea virus; TGEV = transmissible gastroenteritis virus; PRoV = porcine rotavirus.

Table 2. Isolated strains of PEDV and PKV used for sequence alignment and phylogenetic analysis

PEDV			PKV		
Strain name	**GenBank Acc. No.**	**Place of origin**	**Strain name**	**GenBank Acc. No.**	**Place of origin**
JSCZ130818	KM983619	Jiangsu	SH1402	KM983629	Shanghai
HNZK1402	KM983616	Jiangsu	GuaNan4201307	KF977012	Jiangsu
GDGZ1312	KM983611	Guangdong	RuGao2201303	KF977025	Jiangsu
JSBH140210	KM983618	Jiangsu	SiPing26201311	KF977030	Jilin
JSYZ131228	KM983624	Jiangsu	SiPing17201311	KF977029	Jilin
GDGZ13121	KM983612	Guangdong	SiPing15201311	KF977027	Jilin
SH14031	KM983626	Shanghai	SiPing16201311	KF977028	Jilin
HNZY1312	KM983617	Henan	GuanYun13201301	KF977016	Jiangsu
JSTZ1303	KM983622	Jiangsu	HaiZhou4201301	KF977019	Jiangsu
JSYC1303	KM983623	Jiangsu	HN13121	KM983628	Henan
SH1403	KM983625	Shanghai	GuanYun27201301	KF977018	Jiangsu
HNXC1403	KM983615	Henan	HN1312	KM983627	Henan
HNLB1402	KM983613	Henan	Haizhou201301	KF317208	Jiangsu
HNWH1403	KM983614	Henan	GuangZ201312	KJ556981	Jiangsu
JSDF131228	KM983620	Jiangsu	ShuY201312	KJ556982	Jiangsu
JSNT131122	KM983621	Jiangsu			

PEDV = porcine epidemic diarrhea virus; PKV = porcine kobuvirus.

Table 3. Reference strains used for phylogenetic comparisons

PEDV			Kobuvirus		
Strain name	**GenBank Acc. No.**	**Place of origin**	**Strain name**	**GenBank Acc. No.**	**Place of origin**
HNQX	JN400902	China	RBA24	HQ877620	Brazil
HNBF	JN400903	China	WUH1	JQ692069	China
BJ2010	JF690778	China	XX	KC204684	China
DX	EU031893	China	WB-1-HUN	JX177612	Hungary
LZC	EF185992	China	THA/2008	AB624472	Japan
LJB-03	AY608890	China	THA/CMP035	GQ152100	Japan
USA/Colorado/2013	KF272920	USA	K-30-HUN	GQ249161	Hungary
PFF188	FJ687462	Korea	S-1-HUN	NC_011829	Hungary
IA2	KF468754	USA	swine/2007/CHN	FJ493623	China
MN	KF468752	USA	Y-1-CHI	GU292559	China
M2227	FJ687456	Korea	CH/HNXX-4	JX401523	China
Chinju99	DQ845249	Korea	Japan/2009	HM014081.1	Japan
KPEDV-9	AF01588	Korea	CPF4293	HQ234909	Korea
Br1/87	Z24733	England	U-1	AB084788	Japan
CV777	AF353511	Belgium	TB3/HUN/2009	GU245693	Hungary
08RB03	FJ196184	Tailand	Aichi virus 1	AB010145	Japan
M_NIAH1795_04	EU542415	Tailand	US-PC0082	JN088541	USA
M_NIAH2013_95	EU581711	Tailand	M-5/USA/2010	NC_015936	USA
Iowa18984	KF804028	USA			
JS-2004-2	AY653205	China			

PEDV = porcine epidemic diarrhea virus.

co-infections with more than two of the viruses, and PKV was always one of the co-infecting viruses. There were no cases of co-infection with any combination of PEDV, TGEV, and PRoV.

Sequence analysis of the PKV 3D gene

The sequence homology of the 3D gene was assessed for 15 PKV strains collected during the study. Of these,

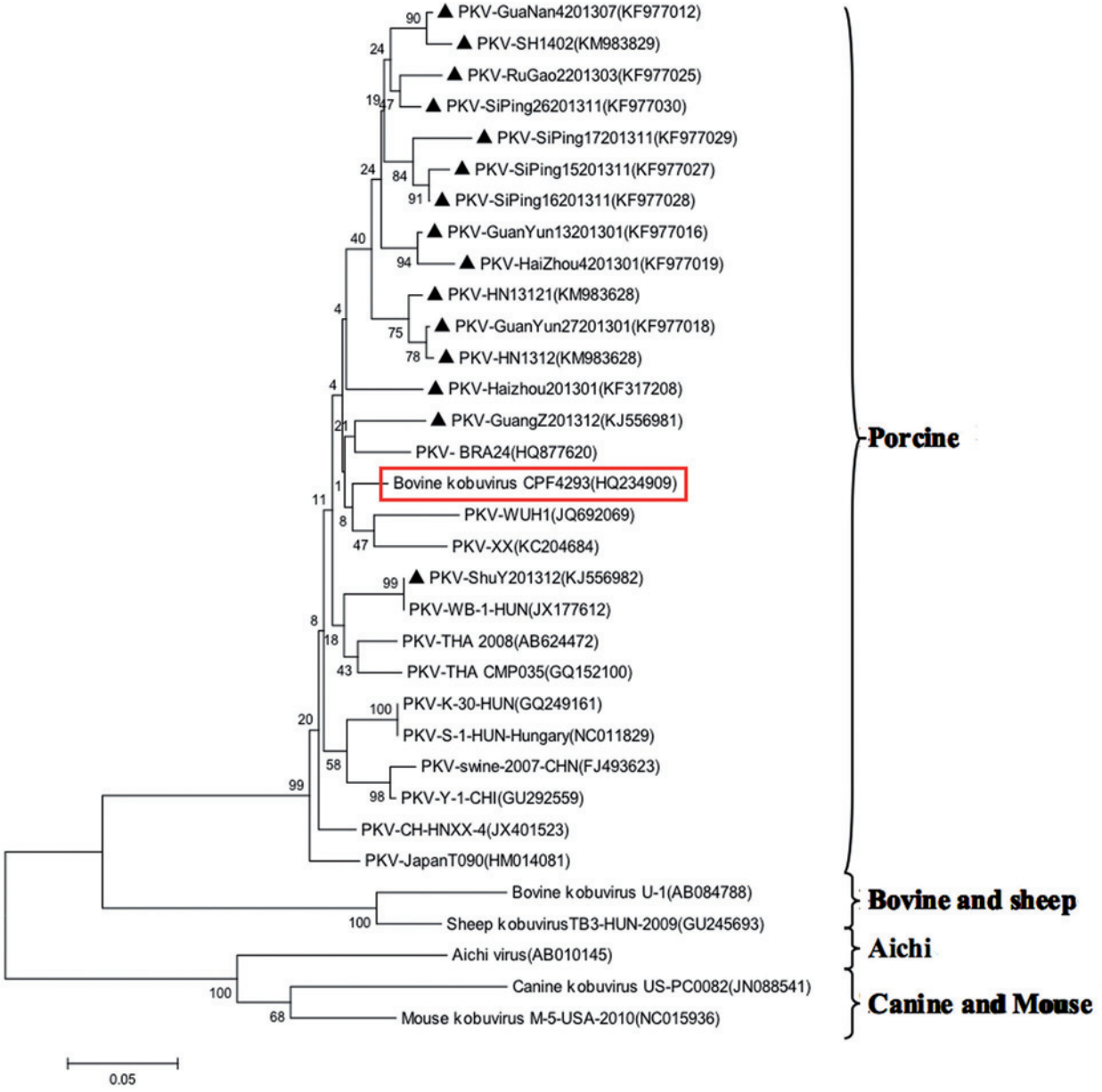

Fig. 1

Phylogenetic analysis by the neighbor-joining method based on partial nucleotide sequences of PKV 3D region (183 bp without primers)
Sequence of 15 PKV strains collected is indicated by the filled triangle.

the amplicons demonstrated 86.8–100% identity on the nucleotide level and 69.4–100% identity on the amino acid level. The nucleotide and deduced amino acid sequence identity of the PKV strains were then compared to reference strains (Y-1-CHI and S-1-HUN) and other kobuviruses. Compared to Y-1-CHI, the nucleotide identity of the collected PKV strains was 90.3–95.9% and the deduced amino acid identity was 66.7–87.5%; compared to S-1-HUN the identities were 88.0–94.9% for nucleotide and 73.6–88.9% for amino acid sequences. Finally compared to other kobuviruses, the nucleotide sequence identity was 69.8–78.8% and the deduced amino acid identity was 27.8–56.9%. In a phylogenetic analysis, 15 strains collected from different geographical locations in China clustered together with the reference PKV strains, while kobuviruses from other species formed two clusters. Interestingly, a bovine kobuvirus clustered with PKV (Fig. 1).

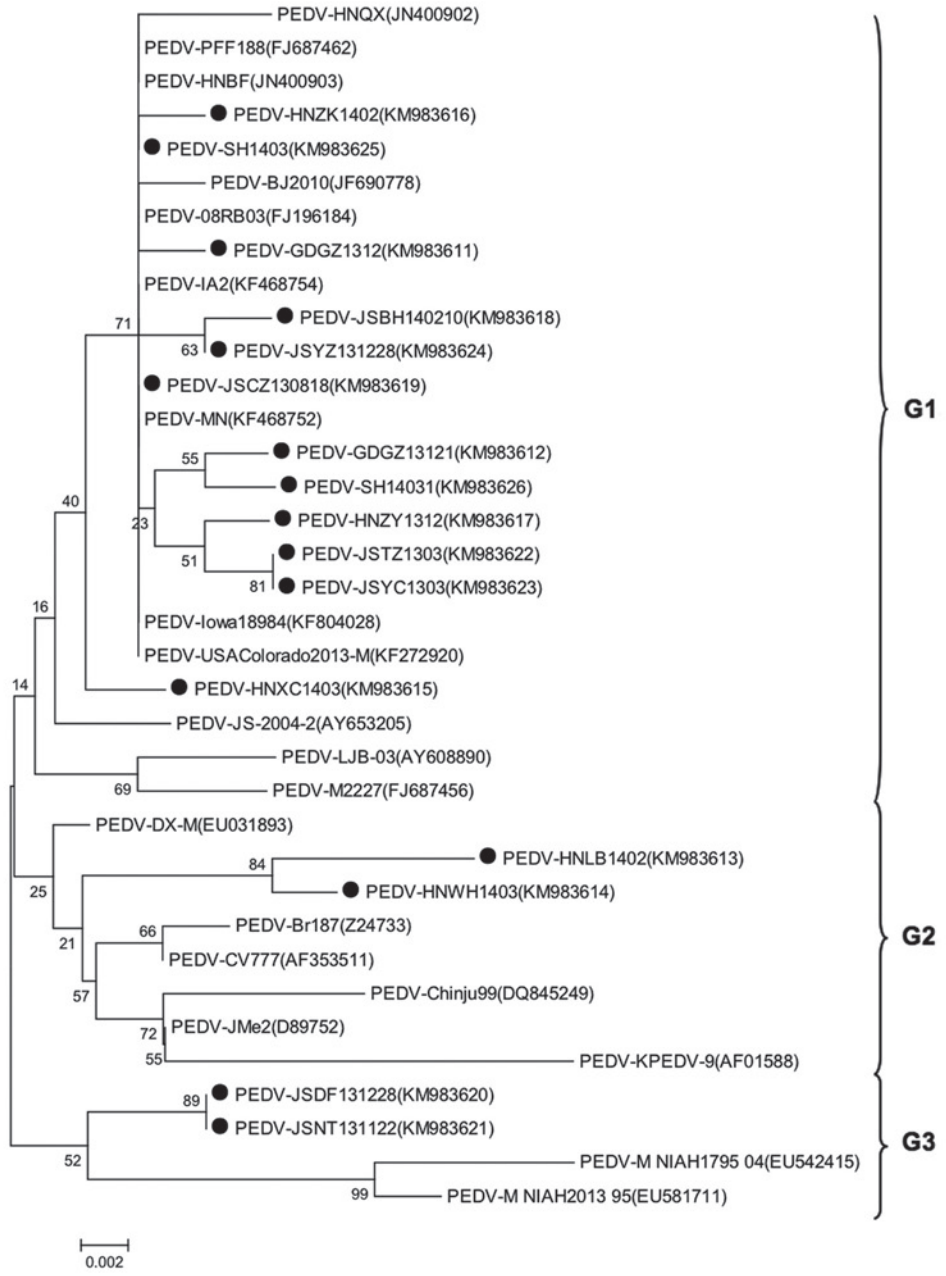

Fig. 2

Phylogenetic analysis by the neighbor-joining method based on the partial nucleotide sequences of the PEDV M region (335 bp without primers)
The sequences of the 16 PEDV collected strains are indicated by the filled circle.

Sequence analysis of the PEDV M gene

The sequence homology of the PEDV strains collected (n = 16) was based on the partial M gene sequence. The 16 amplicons showed 96.8–100% identity at the nucleotide level and 95.9–100% identity at the amino acid level. The nucleotide and deduced amino acid sequences of the collected PEDV strains were then compared to reference strains (CV777 and US) and to other regional PEDV strains. Regarding the reference strains, the nucleotide sequence identity compared to CV777 was 98.1–98.9% and 97.6–100% identity at amino acid level compared to US strains. The deduced amino acid identities of the isolates compared to CV777 and US strains were 97.5–99.2% and 96.7–100%, respectively.

Compared to other regional PEDV strains, the nucleotide sequence identity was 93.5–100% and the deduced amino acid sequence identity was 95.9–100%. Phylogenetic analysis based on the partial M gene sequence resulted in three clusters. The G1 cluster contained 12 PEDV strains collected in China and some reference strains, from the US, Korea, Thailand, and other Chinese regions. Two Henan strains clustered with CV777 in G2 cluster. The G3 cluster contained two Jiangsu strains and two strains from Thailand (Fig. 2).

Discussion

Porcine diarrhea can be caused by PEDV, TGEV, and PRoV, but PEDV was the pathogen responsible for a porcine diarrhea epidemic in China in 2010 (Chen *et al.*, 2012, 2013b; Gao *et al.*, 2013; Ge *et al.*, 2013; Li *et al.*, 2012; Sun *et al.*, 2012), that resulted in major economic losses in the swine industry. The prevalence of PKV ranges from 19.3–99.0% in different countries (Wang *et al.*, 2011), and it can infect pigs of all ages and varieties. PKV has also been linked to porcine diarrhea although its pathogenesis remains unclear (Barry *et al.*, 2011; Chen *et al.*, 2013a). Based on the rates of infection documented in this study, PEDV (24.2%) is still the primary cause of porcine diarrhea in the past two years. The infection rate for PKV is also very high (29.9%), and of the PKV infected samples, 52.1% were co-infections with one other virus (PEDV, TGEV, or PRoV), suggesting a high prevalence of co-infection in the sampled regions. Interestingly, co-infections with PEDV and PKV accounted for 61.8% (47/76) of the PEDV positive samples, which is much higher than the frequency of infection with PEDV alone. Thus, PKV may only be pathogenic as a secondary infection following infection by PEDV or another pathogen (Yang *et al.*, 2014). The high prevalence of co-infection, particularly PKV and PEDV, is a cause for concern and should be seriously considered.

Based on the genetic analyses using the PKV 3D gene, the 15 amplicon sequences had high homology with reference PKV strains at the nucleotide level, but low homology with other reference kobuvirus. This suggests that the PKV 3D gene is highly conserved. Phylogenetic analysis showed that there was no regional difference in PKV strains as the collected strains were distributed in different branches. Although one of the strains (GuangZ201312) clustered with a strain from Brazil (BRA24) and a second strain (ShuY201312) clustered with a strain from Hungary (WB-1-HUN). These findings indicate that the PKVs circulating in China are genetically diverse. The presence of a bovine kobuvirus in the cluster with PKV suggests that there may be interspecies transmission of kobuviruses (Reuter *et al.*, 2010).

The PEDV M gene was also highly conserved based on genetic analyses. All 16 amplicon sequences had high homology with reference PEDV strains from GenBank at both the nucleotide and amino acid levels. PEDV clustered into three groups based on the phylogenetic analysis of the amino acid sequences of the M protein. Recently isolated Chinese PEDV strains tended to vary. Most of the strains in this study belonged to the G1 group. There is likely environmental or immunological pressure on PEDV that is keeping the M gene of the Chinese isolates since 2003 tightly clustered, but distinct from the CV777 vaccine strain. American-like isolates were also in this branch, and the PEDV strains causing outbreaks in America recently may be related to the PEDV strain in China (Tian *et al.*, 2014). Two Henan strains belong to the branch which includes CV777, suggesting that CV777 may be their ancestor. Most notably, two Jiangsu strains clustered with two strains from Thailand, which is quite rare. Therefore, a new genotype may be transmitted to China from other places, and could explain why herds immunized with CV777 continue to experience PEDV outbreaks in China. Thus, additional PEDV vaccines based on different genotypes should be developed to prevent and control this disease.

Conclusions

The rate of infection with PKV and PEDV were very high in different parts of China. The PKV isolates were genetically diverse, while the PEDV isolates were more heterogeneous. Co-infections with PKV and PEDV were very common on pig farms, suggesting that controlling piglet diarrhea might be more complex than previously thought. A better understanding of viruses that cause diarrhea in piglets will aid in better preventing and controlling epidemics of viral piglet diarrhea.

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