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ORIGINAL ARTICLE

Preparation and characterization of an attenuated porcine epidemic diarrhea virus strain by serial passaging

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Abstract

Porcine epidemic diarrhea virus (PEDV) is prevalent in most parts of the world. Owing to its antigenic variation, prevention of the diseases caused by this virus is difficult. In this study, two PEDV isolates with similar growth kinetics were successfully propagated in Vero cells. Complete genome sequence analysis showed that they have a 49nt deletion in the *ORF3* gene and were classified into Group 1, the same group that includes the classical CV777 strain. Recombination analysis revealed that the event had occurred in the *ORF1a* gene, at 3596–6819 nt, among the two PEDV isolates and the CV777 and DR13 strains. During their continuous propagation, 14 nonsynonymous mutations occurred in the *spike (S)* gene of strain JS-2/2014 between generations G5 and G90, but there were no changes between G90 and G100. We assumed that strain JS-2/2014 might be attenuated by the 90th generation. Piglets orally fed with JS-2/2014 G90 showed no clinical symptoms, and no virus was detected in the feces and nasal fluid. In conclusion, JS-2/2014 was successfully identified by screening, was attenuated after propagation in Vero cells, and may serve as a candidate virus for vaccine preparations.

Abbreviations

PEDV	Porcine epidemic diarrhea virus
TGEV	Transmissible gastroenteritis virus
ORF	Open reading frame
S	Spike
N	Nucleocapsid
M	Membrane
Dpi	Days of postinfection

Introduction

Porcine epidemic diarrhea virus (PEDV) is the causative agent of porcine epidemic diarrhea, an enteric disease characterized by acute watery diarrhea, dehydration, and vomiting and which affects pigs of all ages but has a high mortality rate among neonatal piglets [1]. PEDV was first described in England in 1971, and until the end of the 1990s, sporadic cases were reported throughout Europe [2–5]. Since October 2010, severe PEDV outbreaks have occurred in the domestic pig population in China and caused enormous economic losses [6–9].

PEDV vaccines based on the classical PEDV strain CV777, namely the inactivated bivalent transmissible gastroenteritis virus (TGEV) & PEDV vaccine (1999 - present) and the attenuated bivalent TGEV & PEDV vaccine (2003–2006) were widely used in China and played an important role in controlling the disease. Nonetheless, an outbreak of diarrheal disease in 2010 and the high mortality rate among neonatal piglets indicated that the epidemic virus may have changed and escaped the immune response specific to the vaccine [10]. It was subsequently confirmed that variants were responsible for the large-scale outbreak of the disease [11].

The spike (S) protein of coronaviruses performs an important function: binding to cellular receptors and initiating the infection. It also induces neutralizing antibodies *in vivo*. Mutations, including deletions and/or insertions, in the S protein may change the pathogenicity and tissue tropism of coronaviruses; and this mechanism may be the main reason for the virus escaping the immune response generated by vaccination [12]. Therefore, it is necessary to analyze the

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sequence and characteristics of the prevalent PEDV strains for further exploration of these pathogenic mechanisms.

In this study, we first determined the complete genome sequences and biological characteristics of PEDV strains that have emerged in domestic pigs. Subsequently, the genetic relationship between these and other PEDV strains was analyzed. Furthermore, we cultured the virus and screened for attenuated PEDV strains by analyzing the genovariations that occurred during passaging - confirming the results in animal experiments. Our results provide useful insights into the preparation of an effective and safe PEDV vaccine.

Materials and methods

Ethics statement

All animal experiments were conducted under the guidance of the Institutional Animal Care and Use Committee at Centers for Disease Control and Prevention (CDC) and the Laboratory Animal Care International accredited facility.

Origin of the PEDV isolates

Strains JSLS-1/2014 and JS-2/2014 were isolated from the intestinal tissues of piglets with watery diarrhea and were sent to our laboratory for virus detection. Sows were all inoculated with the bivalent killed or attenuated vaccines against TGEV & PEDV. As many as 50% of piglets died within 2 days of birth. Pigs from another farm died at ~1 month of age following the development of watery diarrhea in the clinic.

Preparation of a PEDV inoculum for Vero cells

PEDV-positive fecal samples were diluted 10-fold in PBS and then vortexed briefly, followed by centrifugation at 10,000×*g* for 10 min. The supernatants were passed through 0.22 μm syringe filters and then inoculated onto confluent Vero cells. After adsorption at 37 °C for 1 h, the cells were incubated in Dulbecco's modified Eagle's medium (DMEM). The PEDV strains were identified by RT-PCR and an indirect immunofluorescence assay (IFA).

For the IFA, the Vero cells were fixed with 70% ethanol and incubated with an anti-PEDV monoclonal antibody for 1 h, followed by incubation with a fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG antibody. Finally, cell staining was examined under a fluorescence microscope.

Plaque staining and purification

The PEDV isolates were purified by a plaque method in Vero cells. In brief, confluent cell monolayers in 6-well plates were infected with 10-fold dilutions of each virus isolate (to a total volume of 0.4 mL of PBS) for 1 h at 37 °C. The cells were washed twice with PBS and overlaid with modified Eagle's medium containing 0.9% of agar. The plates were then incubated at 37 °C and 5% CO_2. After 5 days of incubation, the cells were stained with 0.1% crystal violet [13].

Full-length genome sequencing and phylogenetic analysis

Viral RNA was extracted from a 250 μL sample using the TRIzol Reagent (Takara, Shiga, Japan). The PEDV cDNA was synthesized by means of the PrimeScript High Fidelity RT-PCR Kit (Takara). Full-length PEDV genome amplification was performed with primers described previously [14]. The 5′ and 3′ end sequences were determined with the 5′ and 3′ RACE kit (Takara). For each amplicon, more than three independent clones were sequenced to determine the consensus sequence of a given genomic region.

The ClustalX (ver.1.81) software was used to align the full-length genome sequences. Phylogenetic analyses based on the *S* and open reading frame 3 (*ORF3*) genes or the entire genome were performed by the maximum-likelihood method with the general time-reversible nucleotide substitution model, where 1000 bootstrap replicates were implemented in the MEGA6.0 software [15].

Growth kinetics

Growth kinetics of the PEDV strains were measured in Vero cells and compared. The viruses were added at a multiplicity of infection (MOI) of 1 to Vero cells in a 24-well plate. After 1 h, the cell medium was replaced with fresh DMEM containing 2.5 μg/mL trypsin. Culture samples collected at 12, 24, 48, 60, 72, and 96 h postinfection were freeze–thawed twice and then centrifuged at 12,000×*g* for 10 min at 4 °C. The supernatants were collected and analyzed for the virus titer (50% tissue culture infectious dose [$TCID_{50}$]/mL) following the Reed–Muench method [16].

Animal experiments

To evaluate the pathogenicity of a PEDV strain, we analyzed and purchased PEDV-negative pregnant sows. Negativity for known enteric pathogens was confirmed by quantitative RT-PCR. Pigs were orally inoculated with the JS-2/2014 strain (different passages), 1-day-old neonatal piglets were given access to milk and water *ad libitum* (three piglets per group). The virus inoculum was 10^6 $TCID_{50}$ per animal. Clinical symptoms, survival, and virus shedding (daily rectal and throat swabs) were then examined. Virus shedding was quantified by RT-PCR with the following primers, PEDV-F: 5′-TTCCCGTTGATGAGGTGAT-3′, PEDV-R: 5′-AAGCAT

TGACTGAACGACC-3′. Serum and feces samples were collected from each piglet weekly and tested for IgG and IgA antibodies by ELISA.

Results

Isolation of PEDV and its biological characteristics

We isolated six PEDV strains in this study. Due to their high identity, only two strains - JSLS-1/2014 and JS-2/2014 - were chosen for genome analysis. They proliferated successfully in Vero cells without trypsin (Fig. 1a, b) and induced extensive cell death (lysis) without cell fusion (Fig. 1c). The plaque assay confirmed that they had a similar morphology (Fig. 1d). Furthermore, their growth characteristics indicated that they had similar growth kinetics, with replication peaking 72 h after infection (Fig. 1e).

Full-length genome characterization

The genomes of the PEDV isolates were sequenced after the 5th passage. The details for each gene are listed in Table 1. Complete sequence homology analysis of the PEDV isolates and other PEDV reference strains revealed that they shared high identity (99.0%–99.9%) with domestic strains OH851 and FL2013. The phylogenetic tree based on the complete genomic sequences indicated that JSLS-1/2014 and JS-2/2014 isolates clustered in the same group together with classical strains CV777 and DR13 (Fig. 2a).

Next, analysis of the *N*-glycosylation sites within the S, membrane (M), and nucleocapsid (N) proteins indicated

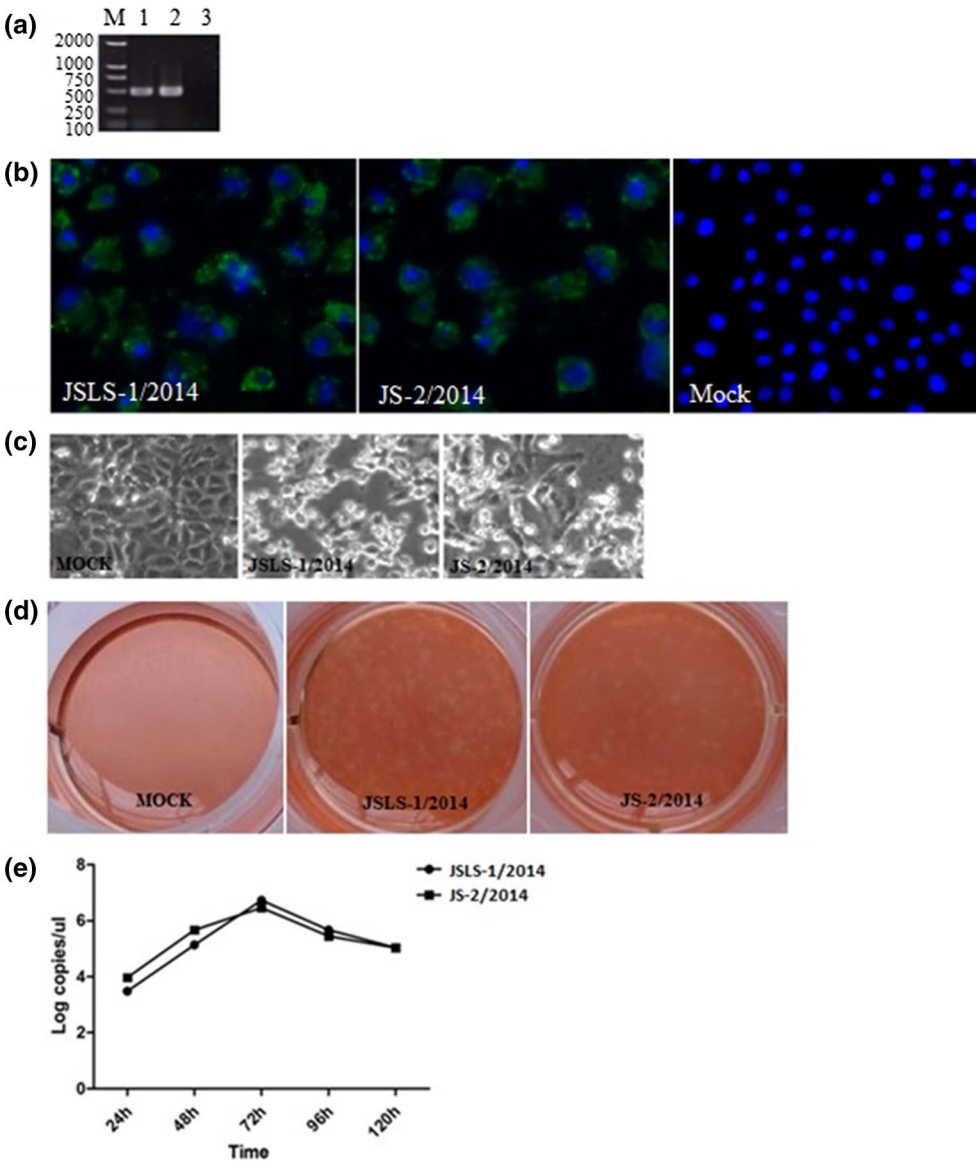

Fig. 1 Cellular characteristics of the two PEDV isolates. PEDV-positive fecal samples were inoculated onto Vero cells in continuous passage culture. (a) The 3rd-passage viruses yielded positive bands of ~550bp in RT-PCR analysis with primers specific to the *M* gene of PEDV. (b) Indirect immunofluorescence assay with DAPI confirmed that PEDV strains JSLS-1/2014 and JS-2/2014 could be recognized by the anti-PEDV monoclonal antibody. (c) After six blind passages, Vero cells infected with the PEDV isolates showed obvious cytopathic effects with widespread death and shedding of the virus. (d) PEDV virus particles were purified by a plaque method, with picking of a single clone each time. The results showed that the plaque morphological features of strains JSLS-1/2014 and JS-2/2014 were similar. (e) Growth kinetics assays indicated that both strains could replicate effectively in Vero cells with similar characteristics

Table 1 Gene information for the porcine epidemic diarrhea virus isolates

Strains	Location of the genes								
	5′-UTR	ORF1a	ORF1b	S	ORF3	E	M	N	3′-UTR
JSLS-1/2014	1–291	292–12576	12576–20540	20537–24685	24685–24960	25291–25500	25508–26188	26200–27525	27526–27869
JS-2/2014	1–292	293–12574	12574–20610	20607–24755	24577–25030	25361–25570	25578–26258	26270–27595	27596–27939

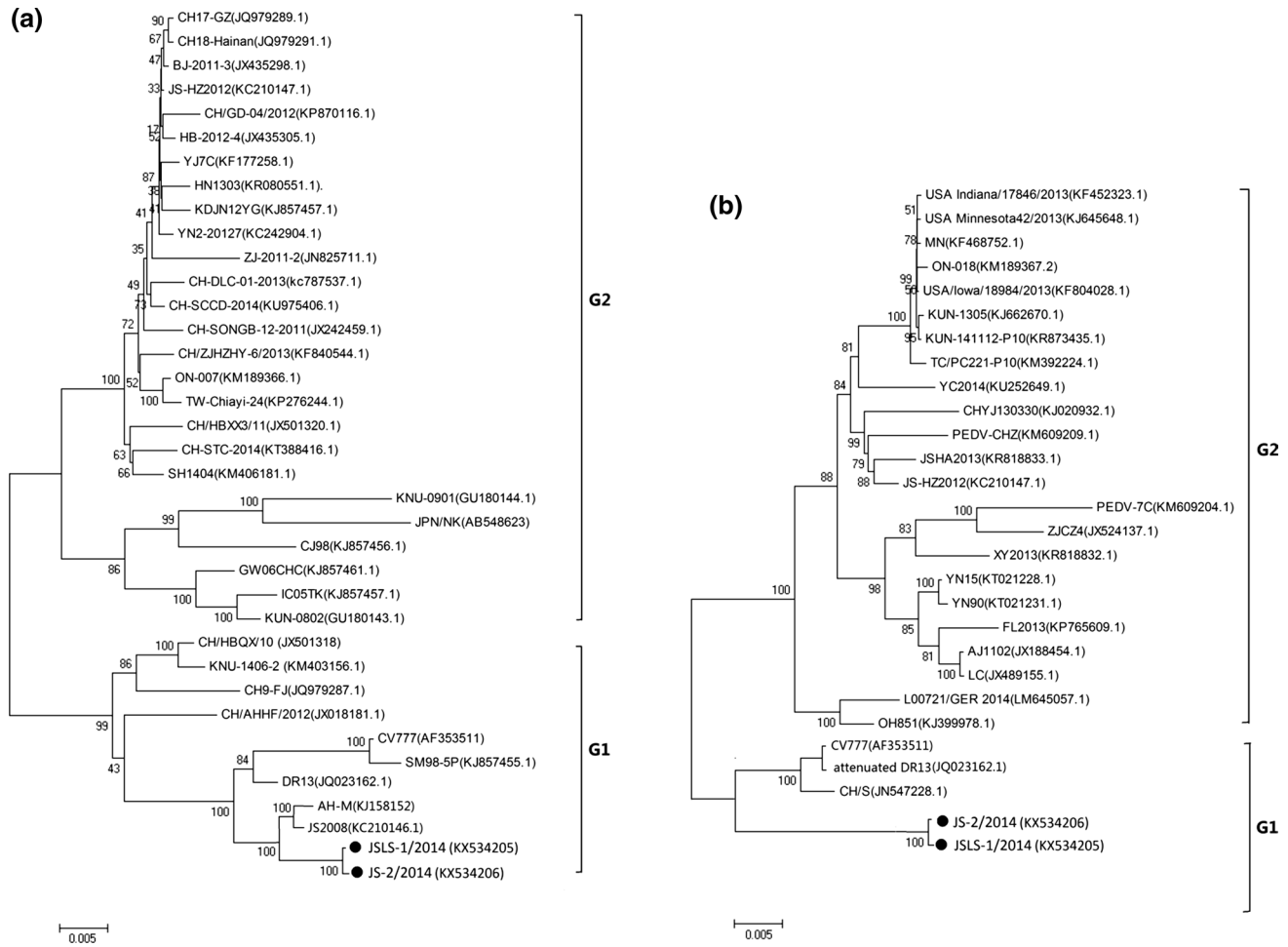

Fig. 2 Phylogenetic analysis based on the nucleotide sequences of the complete genome (a) and *S* gene (b) of our PEDV strains along with reference PEDV strains. The tree was constructed by the neighbor-joining method in the MEGA6.0 software. The numbers at the branches are bootstrap values (%) following 1000 replicates. A dot has been added to the front of the two PEDV strains isolated in this study

that JSLS-1/2014 and JS-2/2014 had the same number of *N*-glycosylation sites in the S protein; one less than the number in the attenuated DR13 strain (Table 2). In the M protein, our two PEDV strains and attenuated DR13 had three *N*-glycosylation sites, whereas CV777 had one more site. In the N protein, all the strains had six *N*-glycosylation sites, except for JSLS-1/2014 which had one less.

Table 2 *N*-glycosylation sites in the PEDV strain *S*, *M* and *N* genes

Genes	N-Glyc			
	JSLS-1/2014	JS-2/2014	attenuated DR13	CV777
S	27	27	28	29
M	3	3	3	4
N	5	6	6	6

Phylogenetic and sequence analyses of genes *S*, *ORF3*, *M*, and *N*

The full *S* sequences of our PEDV isolates and of 33 reference strains from Korea, Germany, the US, and China were compared. The phylogenetic tree, constructed by the neighbor-joining method, showed that the two PEDV isolates from this study clustered within the same group as the classical strains, and genetically distant from the Chinese PEDV strains isolated during 2011–2014 (Fig. 2b). Further comparison indicated that the *S1* genes from our two PEDV isolates were conserved, with amino acid identities of 99.4–99.8%. Nevertheless, they had obvious variation, relative to the domestic PEDV strains isolated before 2014, with amino acid identities of 89.6–91.3%.

Analysis of the *ORF3* genes revealed 99.2% homology in the encoded amino acid sequence between JSLS-1/2014 and JS-2/2014 isolates, whereas with other strains it reached 100% (e.g., AH/HF/2015, HeB/2015/121, and HLJ/QQHR/2015). This meant that there is a certain degree of variation between our two PEDV isolates even though their identity is high. In addition, a 49nt sequence was found to be deleted in the *ORF3* gene in our two PEDV isolates – a feature also found in the attenuated strains DR13, HLJBY, AH-M, and JS2008. This deletion is a typical characteristic of cell-adapted PEDV strains [17]. On the other hand, the *ORF3* gene of other domestic PEDV strains isolated in recent years is intact in the same way as CV777. It was therefore speculated that the pathogenicity of our PEDV isolates was likely attenuated.

The M protein is one of the important proteins of PEDV for activating host immunity. Amino acid comparison indicated that there were only three amino acid mutations between our PEDV isolates, CV777, and the attenuated DR13: at amino acid positions 5, 56, and 167 (data not shown). The sequence between amino acid residues 1 and 50 is a potential epitope, and the fact that most of the site mutations are located in this area implies antigenic diversity.

By analyzing the amino acid sequences of the N protein, we found great differences among the PEDV isolates, CV777, and attenuated DR13. Compared with CV777, strain JS-2/2014 has 14 amino acid mutations (data not shown). Compared with attenuated DR13, strain JS-2/2014 has five amino acid mutations, whereas JSLS-1/2014 has no changes.

Recombination analysis

To determine the involvement of recombination events in the evolution of the isolates, we performed recombination analysis, using the RDP4 software, to compare our two isolates with representative Chinese historical PEDV strains from different clusters in the phylogenetic tree. The findings indicated recombination among the two PEDV isolates and CV777 and DR13 had occurred in the *ORF1a* gene at site 3596–6819 (nt positions; Fig. 3a), with a higher recombination probability being detected for CV777 (Fig. 3b). The

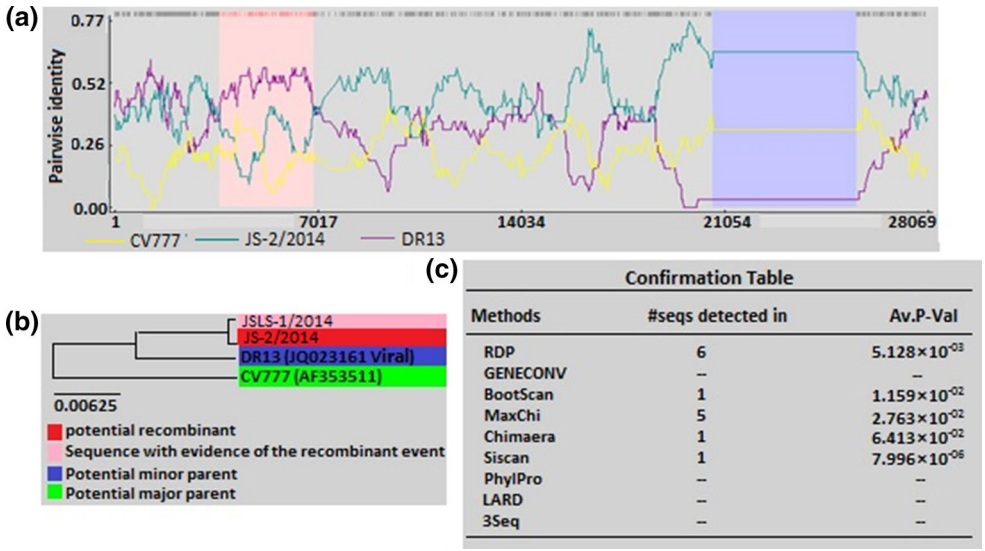

Fig. 3 Recombination analysis of the PEDV isolates. Detection of potential recombination events in the two PEDV strains in the RDP4 software. (a) Four major recombination breakpoints were located, respectively, in genes *ORF1a* (nt3596 and nt6819), *S* (nt20743), and *M* (nt25758). The analysis was performed with an F84 distance model, a window size of 1000 bp, and a step size of 200 bp. (b) Evolutionary relations among the two PEDV strains and the putative parental strains on the basis of full-length genomes. Phylogenetic trees were generated by the distance-based neighbor-joining method in the RDP4 program. (c) Scores of the recombination events detected by the nine methods in RDP4

average P values for JSLS-1/2014 and JS-2/2014 were 3.677 × 10^{-3} and 9.426 × 10^{-3}, respectively (Fig. 3c).

Genovariation during cell propagation

It has been reported that the PEDV *S* gene demonstrates the biggest variability during evolution, prompting us to explore its key genovariation during cell propagation. These data will provide important information for researchers regarding cellular adaptation, virus attenuation, and pathogenicity.

Strain JS-2/2014 was serially passaged in Vero cells, and the 5th (G5), 20th (G20), 50th (G50), and 90th (G90) generations of the virus were selected and sequenced. Comparison of the amino acid sequences revealed that there were 14 missense mutations in the *S* gene (Table 3). Three amino acid residues (at sites 288, 330, and 973) were mutated by G20, six (at sites 360, 784, 816, 901, 983, and 1033) more were mutated at G50, and five more (at sites 15, 56, 132, 1204, and 1308) were mutated by G90. It is worth noting that the amino acid variations at passages G20 and G50 also participated in the DR13 attenuation process. Nevertheless, the five other amino acid mutations were not seen in other PEDV strains and may be specific to strain JS-2/2014.

The JS-2/2014 G90 strain was not pathogenic to piglets munity

Strains JS-2/2014 G20, JS-2/2014 G60, and JS-2/2014 G90 at a titer of 10^6 $TCID_{50}$ were orally fed to PEDV antibody-negative weaned piglets. After the virus challenge, the piglets fed with strain JS-2/2014 G90 appeared asymptomatic, and no virus was detected in the feces and nasal fluid. By contrast, piglets infected with the JS-2/2014 G20 strain developed watery diarrhea and died at 5 days post-infection (dpi) (Fig. 4a). In addition, the piglets infected with the JS-2/2014 G60 strain did not develop diarrhea but had to be detoxified for a period (Fig. 4b). We found that piglets inoculated with JS-2/2014 G60 grew slowly before 6 dpi, whereas the weight gain of piglets inoculated with JS-2/2014 G90 was normal relative to that of control piglets (Fig. 4c). Furthermore, infection with JS-2/2014 G90 activated strong IgG and IgA antibody responses (Fig. 4d). These results confirmed that the JS-2/2014 G90 strain was attenuated, and this change was attributed to its repeated propagation in tissue culture.

Discussion

Porcine diarrheal disease has become epidemic in China in recent years, causing huge financial losses in the pig industry [18]. PEDV, TGEV, and porcine rotavirus (PoRV) are the three main pathogens of this disease [19, 20]. To trace the epidemiology of the recent outbreaks, porcine fecal samples were collected; our results revealing that PEDV had a higher infection rate than both TGEV and PoRV. This result is consistent with data from other reports [21, 22].

To explore the evolution and pathogenicity of this virus, we next identified two PEDV variants in porcine fecal samples from the Shanghai and Jiangsu provinces. Of note, unlike the PEDV variants isolated in China in recent years, our two isolates could propagate in Vero cells successfully without trypsin. Nevertheless, most of the PEDV variants isolated in recent years can proliferate in mammalian cells successfully only in the presence of trypsin [23]. It is unknown whether this phenomenon is related to the *S* gene, which is important for cell adaptation [24], or to the *ORF3* gene, which is a recognized marker for attenuated and virulent strains [25].

Our sequence analysis revealed that these two isolates clustered in the same group as classical strain CV777, and all had a 49nt deletion in the *ORF3* gene. Sun *et al*. [14] reported that all newly isolated strains had intact ORFs that could yield translation of a 224aa protein. These studies suggest that this area of the genome may be involved in determining cell tropism and pathogenicity of the virus [26]. Park *et al*. [27] analyzed a cell culture-adapted PEDV (passage 100) strain with a smaller *ORF3* gene and found it to have lower virulence relative to the wild-type virus. This may mean that the pathogenicity of our two PEDV isolates is different from that of the PEDV variants isolated in recent years.

The S protein of PEDV has always been used as a marker of viral variation. Under the pressure of herd immunity, the *S* gene of PEDV mutates frequently, with some of the missense mutations altering viral antigenicity to aid in the

Table 3 Genovariation of the *S* gene in the JS-2/2014 strain during propagation

Strains of different passage number		Location of the amino acid													
		15	56	132	288	330	360	784	816	901	973	983	1033	1204	1308
JS-2/2014	G5	S	G	I	L	S	L	T	F	R	F	Y	K	H	Q
	G20	S	G	I	W	F	L	T	F	R	V	Y	K	H	Q
	G50	S	G	I	W	F	K	M	V	G	V	H	N	H	Q
	G90	L	M	N	W	F	K	M	V	G	V	H	N	Y	R
	G100	L	M	N	W	F	K	M	V	G	V	H	N	Y	R

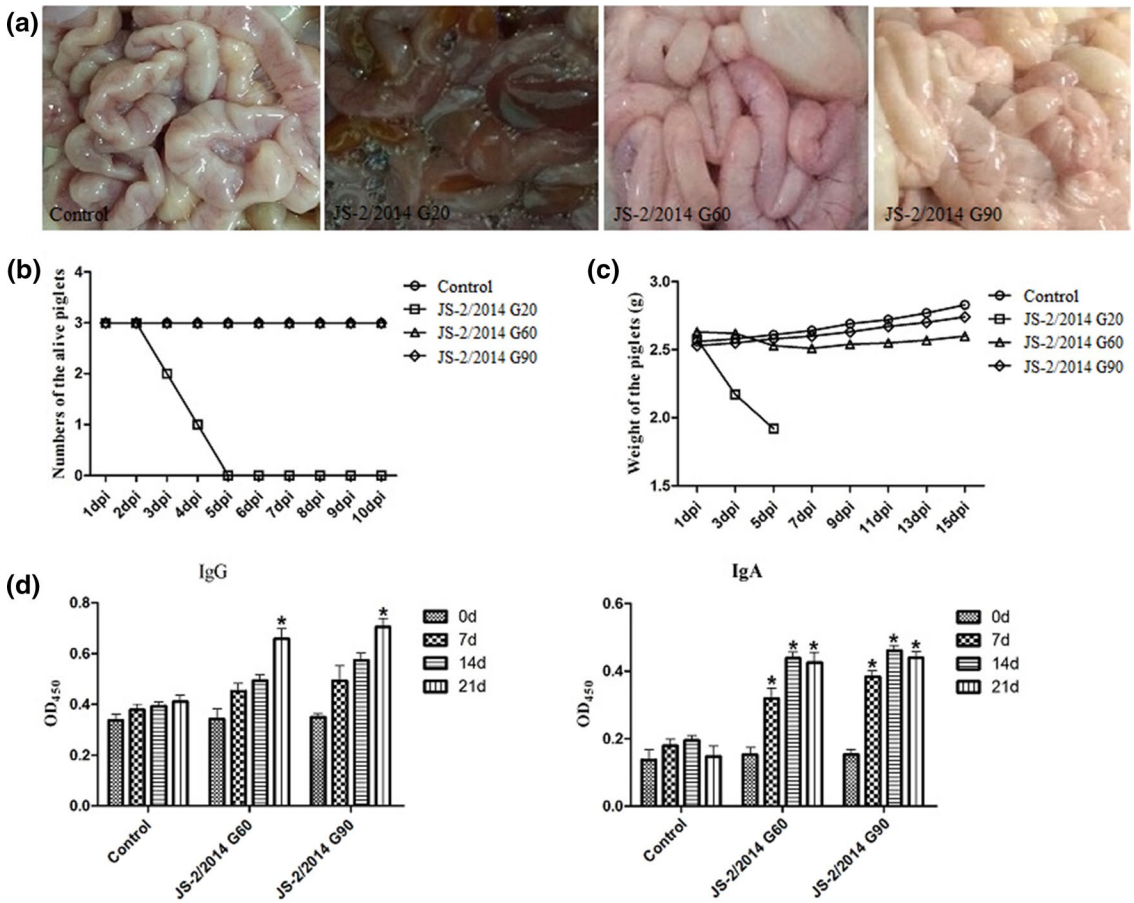

Fig. 4 Pathogenicity assays for a PEDV strain in piglets. Eight piglets were distributed into four groups and fed with 1mL of PBS, JS-2/2014 G20, JS-2/2014 G60, or JS-2/2014 G90, respectively. Piglets fed with JS-2/2014 G20 died within 5 days and had severe intestinal lesions, while the other piglets were alive with no lesions in their intestines (a and b). (c) Strains JS-2/2014 G60 and JS-2/2014 G90 had only a weak effect on piglet weight gain. Piglets fed with strain JS-2/2014 G90 manifested better weight gain than those fed with JS-2/2014 G60. (d) Oral immunization with JS-2/2014 G90 gave piglets high levels of IgG and IgA antibodies

virus's escape from preexisting immunity. Thus, periodic vaccine updates may be required to ensure sufficient efficacy against emerging virus variants [28]. In the present study, our data revealed that there is little variation in the *S* gene among the two PEDV isolates, and that they are most similar to the attenuated DR13 strain. To further explore the role of the *S* gene in virulence, the JS-2/2014 strain was successively cultured, and *S* gene variation during cultivation was analysed. Of note, we found that there were three amino acid mutations between JS-2/2014 G5 and JS-2/2014 G20, six between JS-2/2014 G20 and JS-2/2014 G50, and five between JS-2/2014 G50 and JS-2/2014 G90. It is worth noting that the amino acid variations between JS-2/2014 G5 and JS-2/2014 G50 were also identified in the DR13 attenuation process. This finding indicates that these missense mutations in the *S* gene may be related to viral pathogenicity. Furthermore, animal experiments confirmed that piglets orally fed JS-2/2014 G5 (10^6 $TCID_{50}$) had mild diarrhea and long-term excretion. On the other hand, piglets challenged with JS-2/2014 G90 (10^6 $TCID_{50}$) appeared asymptomatic, with no virus detected in the feces and nasal fluid, indicating that G90 was attenuated and therefore is potentially safe for vaccine preparations. Moreover, recombination analysis confirmed that this strain has a high probability of being recombined with CV777 and DR13. Most recently, the CH/HNQX-3/14 strain was reported as a CV777 and DR13 strain recombinant with the highly virulent CHN/ZMDZY/11 strain. Not surprisingly, this strain was detected on a swine farm that is located in the same province as the CHN/ZMDZY/11 strain; both CV777 and DR13 PEDV vaccines were used on this farm [29]. Therefore, it is possible that the virulence-enhanced CV777 and DR13 strains had been present in some areas, resulting in virus recombination.

In conclusion, this study investigated the epidemiology behind recent outbreaks of porcine diarrheal disease in China and identified two PEDV isolates from Shanghai and Jiangsu. Furthermore, we successfully obtained an attenuated PEDV strain by screening. The results should help our

understanding of the evolution of PEDV and offer a suitable attenuated strain for vaccine preparation.

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Compliance with ethical standards

Conflict of interest There are no conflicts of interest associated with this article.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Furthermore, this article does not contain any experiments with human subjects or animals performed by any of the authors.

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