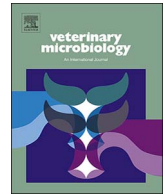Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Genetic and pathogenic characterization of a novel reassortant mammalian orthoreovirus 3 (MRV3) from a diarrheic piglet and seroepidemiological survey of MRV3 in diarrheic pigs from east China

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ARTICLE INFO

ABSTRACT

Mammalian orthoreoviruses (MRVs), which cause gastrointestinal and respiratory illness, have been isolated from a wide variety of mammalian species including bats, minks, pigs and humans. Here we report the isolation and genetic and pathogenic characterization of a novel MRV type 3 (MRV3), named MRV-ZJ2013, from the diarrheic feces of piglets in Zhejiang province, China. Genomic and phylogenetic analysis shows that MRV-ZJ2013 may have originated from reassortments among mink, bat, and pig MRVs, suggesting the hypothesis that interspecies transmission has occurred in pig herds. Neonatal piglets infected with MRV-ZJ2013 displayed mild clinical signs such as poor appetite and soft feces, but vomiting and diarrhea were not observed. Fecal virus shedding was detected only in three out of six piglets, each for one- or two-day post-infection. In contrast, piglets inoculated with a virulent porcine epidemic diarrhea virus (PEDV) strain as the control group had severe signs characterized by acute vomiting and watery diarrhea. These findings suggest that the virulence of MRV-ZJ2013, if any, was likely not significant compared to that of PEDV. A seroepidemiological survey of MRV by means of an indirect enzyme-linked immune-sorbent assay (ELISA) based on a recombinant MRV3 capsid protein sigma1 as antigen revealed a high seroprevalence (77%) in 1037 samples from diarrheic pigs of different ages from 24 herds in seven provinces of east China between 2015 and 2016, indicating that MRV3 is endemic in pig herds in China, and may contribute collectively to enteric disease along with other porcine pathogens.

1. Introduction

Diarrhea is a common disorder in pigs, and the associated dehydration is a leading cause of mortality among piglets, leading to substantial economic losses in China, South Korea and the United States. The etiology of diarrhea is varied, including multiple viral, bacterial, and parasitic pathogens, but viruses such as porcine epidemic diarrhea virus (PEDV) (Huang et al., 2013), porcine delta coronavirus (PDCoV) (Jung et al., 2015; Wang et al., 2015b), transmissible gastroenteritis virus (TGEV) (Kim et al., 2000), rotavirus (RV) (Santos and Hoshino, 2005) and other enteroviruses (EV) (Saif, 1999), are the predominant factors in most cases. Repeated outbreaks have also been reported on farms in which piglets had been immunized by vaccines targeting these viruses. Recently, mammalian orthoreovirus (MRV), which can cause

diarrhea alone or in co-infections with other known pathogens, has been of great concern in Asia, especially in China and South Korea (Dai et al., 2012a; Kwon et al., 2012a; Zhang et al., 2011b).

Orthoreoviruses belong to the genus *Orthoreovirus* in the family *Reoviridae*, and are divided into two subgroups, fusogenic and non-fusogenic, based on their ability to induce cell–cell fusion and syncytium formation (Day, 2009). Members of the species MRV are nonfusogenic, and are classified into four major serotypes (type 1 Lang, type 2 Jones, type 3 Dearing, and type 4 Ndelle) (Day, 2009). The MRV dsRNA genome contains 10 segments divided into three size classes based upon their characteristic mobility in gel electrophoresis: three large segments (L1, L2 and L3), three medium segments (M1, M2 and M3), and four small segments (S1, S2, S3 and S4). The total genome size is approximately 23,500 base pairs (Mertens, 2004).

MRVs were traditionally believed to be the causative agents of mild respiratory and enteric infections, without significant clinical impact. However, in the last decade, increasing numbers of studies in humans and other mammals have shown that they can cause severe illness in humans and other mammals, including upper respiratory tract infections, encephalitis, and diarrhea (Chua et al., 2008; Ouattara et al., 2011a; Steyer et al., 2013a). MRVs have been isolated from a broad range of mammalian species, including bats (Hu et al., 2014; Kohl et al., 2012; Lelli et al., 2013; Lorusso et al., 2014; Thalmann et al., 2010; Wang et al., 2015a; Yang et al., 2015), civet cats (Li et al., 2015), cows (Anbalagan et al., 2014), mink (Lian et al., 2013), pigs (Dai et al., 2012b; Kwon et al., 2012b; Thimmasandra Narayanappa et al., 2015; Zhang et al., 2011a), dogs (Decaro et al., 2005) and humans (Chua et al., 2008; Ouattara et al., 2011b). However, porcine MRV failed to arouse the concern of researchers until 2007, when it was first reported as the cause of diarrhea in newborn piglets in China (Zhang et al., 2011a). Serotypes 1 and 3 of porcine MRV were subsequently isolated in China (Dai et al., 2012b; Zhang et al., 2011a) and the prevalence and genetic diversity of porcine MRVs circulating in South Korea was assessed in 2012 (Kwon et al., 2012b). They found that 19.0% of diarrheic fecal samples collected from 78 pig farms around the country tested positive for porcine MRV (Kwon et al., 2012b). In 2015, a novel type 3 MRV (MRV3) was isolated and characterized in the USA, having caused severe diarrhea and acute gastroenteritis in neonatal piglets with 100% mortality by 3 days post-infection (Thimmasandra Narayanappa et al., 2015). Therefore, porcine MRV may contribute to enteric disease alone or in combination with other swine pathogens.

Type 2 MRVs (MRV2) have recently been isolated, possibly originating from reassortment between bat, pig, and/or human MRV strains which have been associated with diarrhea, acute gastroenteritis and necrotizing encephalopathy (Wang et al., 2015a). Six bat MRV strains have been isolated which share high S1 segment sequence similarity with MRVs recovered from diseased mink, piglets, or humans. These studies suggest that interspecies transmission can occur between bats and pigs (Yang et al., 2015), with alteration of viral characteristics upon intragenic rearrangement and reassortment of the reovirus genomes. Here, we describe a porcine MRV3 strain with evidence of reassortment between mink, bat and pig viruses isolated from the diarrheic feces of piglets in Zhejiang province, China.

2. Materials and methods

2.1. Virus isolation

Vero (African green monkey kidney) cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and 1% antibiotics (penicillin, streptomycin, w/v). Fecal specimens, collected from 5 day-old diarrheic pigs in Zhejiang province in 2013, were homogenized in DMEM containing antibiotics, centrifuged at $4000 \times g$ for 15 min, and the supernatants were collected. Samples were used to inoculate confluent monolayers of Vero cells with 0.5% (w/v) trypsin at 37 °C and 5% CO_2 and observed daily for 7 days to track development of cytopathic effect (CPE). The unknown virus was adapted and passaged 5 times serially using the culture supernatant in Vero cells then subjected to the next generation sequencing on an Illumina MiSeq platform by a commercial company (Huada Gene Technology Co., Ltd.). Briefly, random RT-PCR was performed using first reverse transcription and then primer extension using Klenow DNA polymerase and a primer with degenerate 3′-end (GCCGACTAATGCGTAGTCNNNNNNNNNN). The double stranded DNA was further amplified and the PCR product was then used as input to generate a library for Illumina MiSeq (2×250 bases) using Nextera™ XT Sample Preparation Kit with dual barcoding. The isolated virus was named MRV-ZJ2013 after determination of the genome.

2.2. Plaque assay and generation of MRV-ZJ2013 virus stock

Monolayers of Vero cells grown to 90% confluency in 6-well plates were inoculated with 10-fold serial dilutions of MRV-ZJ2013 suspended in modified Eagle's medium (MEM) supplemented with 0.5% (w/v) trypsin. The virus was allowed to adsorb to the cell monolayer by incubating 2 h at 37 °C followed by removal of the inoculum then 2 ml of agar overlay (1% agar in MEM supplemented with 1% penicillin/streptomycin and 0.5% trypsin) was added to each well and allowed to solidify at room temperature for 10 min. After incubation at 37 °C for 2 days, cells were fixed by 2% formaldehyde solution and stained with crystal violet for visualization of plaques. The plaque-purified MRV-ZJ2013 was propagated in Vero cells as described above, with virus particles harvested from cells by three freeze-thaw cycles and the resulting suspension purified from cell debris by low-speed centrifugation ($4000 \times g$ for 15 min) used as the virus stocks for the subsequent study. The titer of the virus stock was determined by the plaque assay.

2.3. Electron microscopy

Vero cells infected by the MRV-ZJ2013 (at 6, 12, and 24 h post-inoculation, hpi) were fixed with 2.5% glutaraldehyde in phosphate buffer (0.1 M, pH 7.0) and 1% OsO4 in phosphate. Specimens were dehydrated in a graded series of ethanol dilutions (30%, 50%, 70%, 80%, 90%, 95% and 100%) for 15–20 min at each step, then transferred to absolute acetone for 20 min. Subsequently, the specimens were placed in one of three mixtures of absolute acetone and Spurr resin (1:1, 1:3, and pure Spurr resin) for 1 h, 3 h, and overnight, respectively. Finally, ultrathin sections were stained by uranyl acetate and alkaline lead citrate for 5–10 min and observed using a Hitachi Model H-7650 TEM.

2.4. Characterization of in vitro growth and physicochemical properties of MRV-ZJ2013

Viral growth kinetics were examined by infecting Vero cells with MRV-ZJ2013 at an MOI (multiplicity of infection) of 0.01 for 2 h at 37 °C, after which the inoculum was replaced by maintenance medium. Supernatants of infected cells after freeze-thaw cycles were collected at 0, 6, 12, 24, 36, 48, 60, and 72 hpi, and virus titers ($TCID_{50}$) at each time point were determined in triplicate on Vero cells. UV-inactivated MRV-ZJ2013 was used as a negative control. Temperature sensitivity was assayed; MRV-ZJ2013 was heated to 37, 55, 65, 80, and 100 °C for 1 h, and infectious titers ($TCID_{50}$) were determined in triplicate on Vero cells.

2.5. Sequence and phylogenetic analysis

Total RNA was extracted from the isolated virus with TRIzol reagent according to the manufacturer's instructions (Invitrogen), and cDNAs were amplified with specific primers for each of the 10 segments according to the manufacturer's instructions (SuperScript II, Invitrogen). A total of 14 primer pairs were designed to amplify the complete genome of MRV-ZJ2013 (the primer sequences will be made available upon request). The RT-PCR products were individually subcloned into a pCR-Blunt vector (Invitrogen) followed by Sanger sequencing to determine the consensus sequences. The nucleotide and deduced amino acid (aa) sequences of the L class (L1, L2 and L3), M class (M1, M2 and M3) and S class (S1, S2, S3, and S4) segments were compared with the sequences available in GenBank using Mega5.2 program. Evolutionary analysis was performed using the maximum likelihood method and the Jukes-Cantor evolutionary model, with bootstrap consensus trees inferred from 1000 replicates taken to represent the evolutionary history of the taxa analyzed.

2.6. Generation of anti-sigma1 (σ1) rabbit polyclonal antibodies

The S1 gene encoding the viral outer capsid protein σ1 was cloned into the pET-22b (+) vector (Novagen), and the recombinant plasmid was verified by DNA sequencing. Plasmids were transformed into Rosetta (DE3) competent cells, and induced by 1 mM IPTG for 5 h. The bacteria were collected by centrifugation ($6000 \times g$, 15 min), then lysed via the supersonic schizolysis method and analyzed by SDS-PAGE. Protein was purified by the Ni-NTA His•Bind® Resin system (Transgen tech, DP101, Beijing, China) and used to immunize two rabbits to produce the anti-σ1 polyclonal antibody. Indirect immunofluorescence analysis (IFA) of MRV3-infected Vero cells was used to test the reactivity of the antibody. A mouse monoclonal antibody directed against type 3 orthoreovirus σ1 protein (clone 2Q2048; Abcam, United States) was used as a positive control.

2.7. Pathogenesis study in neonatal pigs

A pathogenesis experiment was approved by the Experimental Animal Ethics Committee of Zhejiang University (no. ZJU20170026). Eighteen 3-day-old conventional piglets, RNA negative for MRV3, PEDV, PDCoV, TGEV, and RV in the fecal samples, were assigned into three groups with 6 in each. Piglets in group A were each received 4 ml of DMEM orally as negative controls, whereas piglets in groups B and C were challenged orally with a PEDV virulent strain ZJU/G2/2013 (GenBank accession no. KU558701) at 1×10^5 plaque-forming unit (PFU)/ml (4 ml per pig) and MRV-ZJ2013 at 6×10^6 PFU/ml (4 ml per pig), respectively. The challenge dosage of MRV-ZJ2013 in group C referred to a recent study (Thimmasandra Narayanappa et al., 2015). All pigs were monitored daily for rectal temperature and clinical signs. Four piglets in each group were euthanized for histological examinations by hematoxylin and eosin (HE) staining at 3, 5, 7 and 14 days post-infection (dpi) with one piglet at each time point, respectively. Fecal swabs for viral RNA detection were collected at 0, 1, 2, 3, 4, 5, 7, and 14 dpi from all remaining pigs, while serum samples that were tested for anti-MRV3 IgG by an enzyme-linked immunosorbent assay (ELISA; described below) were collected at 0, 3, 5, 7 and 14 dpi. Fecal virus shedding was tested by a nested RT-PCR targeting the L1 gene. The first-round PCR was performed with the primers L1/F1 (5′- TTC-ACTCAGGCATTATCCGA-3′) and L1/R1 (5′- TCCGCTTCTGACTCCTGA-3′) in a total volume of 50 μl. A 5-μl aliquot of the first-round PCR product was used for the second-round PCR with the primers L1/F2 (5′-GGTCGTAGTGAGTGGGAGA-3′) and L1/R2 (5′-ACTGCGCTGTTAATG-CTTTC-3′). Immunohistochemistry (IHC) detection of MRV3-specific antigen with the rabbit anti-σ1 antibody was performed on formalin-fixed sections of small intestine tissues as previously described (Zhang et al., 2015).

2.8. Serologic assays

An indirect ELISA based on the recombinant purified MRV σ1 protein as antigen was developed. The optimal concentrations of the antigens and the optimal dilutions of sera and horseradish peroxidase (HRP) conjugates were determined by checkerboard titrations. The optimal amount of the σ1 protein was 0.387 ng per well. The optimal ELISA results were obtained by using a 1:100 dilution of serum samples and a 1:2000 dilution of IgG conjugates. The purified σ1 proteins were diluted in carbonate coating buffer (pH 9.6), which was used for coating 96-well ELISA plates with 100 μl/well. After incubation at 37 °C for 2 h, each well was washed 3 times with 300 μl of PBS (phosphate buffered saline)-T washing buffer (0.01 M PBS, 0.05% Tween-20, pH 7.4) and blocked with blocking buffer (Thermo Fisher Scientific, USA) in a volume of 300 μl for 1 h at 37 °C. One hundred microliters of each diluted serum sample was transferred into the corresponding well on the ELISA plates and incubated at 37 °C for 2 h. After the wells were washed with PBS-T, the diluted HRP-conjugated goat anti-swine IgG

(Thermo Fisher Scientific, USA) was added to each well in a volume of 100 μl, and the plate was incubated at 37 °C for 1 h. A volume of 100 μl of TMB Color liquid (Solarbio, Beijing, China) was added to each well and incubated for 10 min at room temperature. The reaction was stopped by the addition of 50 μl/well of 2 M sulfuric acid. The plates were then read at 450 nm by using a spectrophotometer. All serum samples were run in triplicates. Positive and negative controls were screened as described previously (Huang et al., 2011) by serum western blot using the purified σ1 protein as antigen. The ELISA cutoff value was calculated as the mean OD value of the negative control group (n = 4) plus three times of the standard deviation. Samples with an OD value above the cutoff were considered as being positive. For simplification, a cutoff of 0.32 was used across plates to separate seronegative and seropositive samples when simultaneously evaluating.

The MRV3-specific virus-neutralizing (VN) antibody titers were determined after reacting serial 2-fold dilutions of the selected serum samples with 100 TCID$_{50}$ of MRV3-ZJ2013 for 60 min at 37 °C. Eight Vero cell monolayers per dilution on 96-well microplates were inoculated and then examined for CPE at 2 dpi. VN titers were calculated using the Reed and Muench method and expressed as the reciprocal of the highest serum dilution giving 50% CPE. The cut-off value was determined with 1:4. Representative serum samples were selected for comparisons between the ELISA and VN tests and determining the correlation.

2.9. Seroepidemiological survey

A total of 1037 serum samples collected from diarrheic pigs between 2015 and 2016 was analyzed by the σ1-based indirect ELISA. These serum samples were taken from 24 commercial herds in the Shandong, Henan, Jiangxi, Hunan, Jiangsu, Heilongjiang and Zhejiang provinces from east China, respectively. There were 382 samples from sows (86 gilts, 76 primiparous sows, and 220 multiparous), 152 from 1 week-old piglets, 123 from 3 week-olds, 69 from 5 week-olds, 50 from 7 week-olds, 115 from 11 week-olds, 100 from 17 week-olds, and 46 from 20 week-olds.

2.10. Data analysis

All data were processed using SPSS software (version 20.0) and GraphPad Prism program similar to the procedures described previously (Huang et al., 2012). The *t*-test was used to evaluate the statistical significance of the virus titers and the values of OD450 in ELISA. Analysis of variance (ANOVA) was used for assessment of mean clinical scores in different treatment. Statistical significance was set to a *P*-value of 0.05.

3. Results

3.1. Identification of a novel MRV from a diarrheic piglet in Vero cells

The initial aim of the study was to isolate the variant PEDV strains in blind-passaged Vero cells from the PEDV RNA-positive diarrheic feces of piglets. Unexpectedly, an unknown viral agent inducing cell lysis without forming syncytia was identified in one of the samples. After 5 passages with virus isolated from fecal suspension supernatants (MRV-ZJ2013), Vero cells showed CPE that was characterized by enlarging and rounding of the infected cells and then detachment after 20–30 hpi (Fig. 1A). Infected cell monolayers were completely detached by 72–96 hpi (Fig. 1B) whereas the mock-infected cells grew normal (Fig. 1C). Virus plaques were observed at 2 dpi (Fig. 1D). Electron microscopy of negatively stained virus-infected Vero cells (12 and 24 hpi) demonstrated the presence of icosahedral non-enveloped viral particles ranging in size from 70 to 90 nm that arranged neatly in the cytoplasm of the infected cells (Fig. 1E–F). Since we could not identify the virus by means of PCR or RT-PCR for detection of common viruses
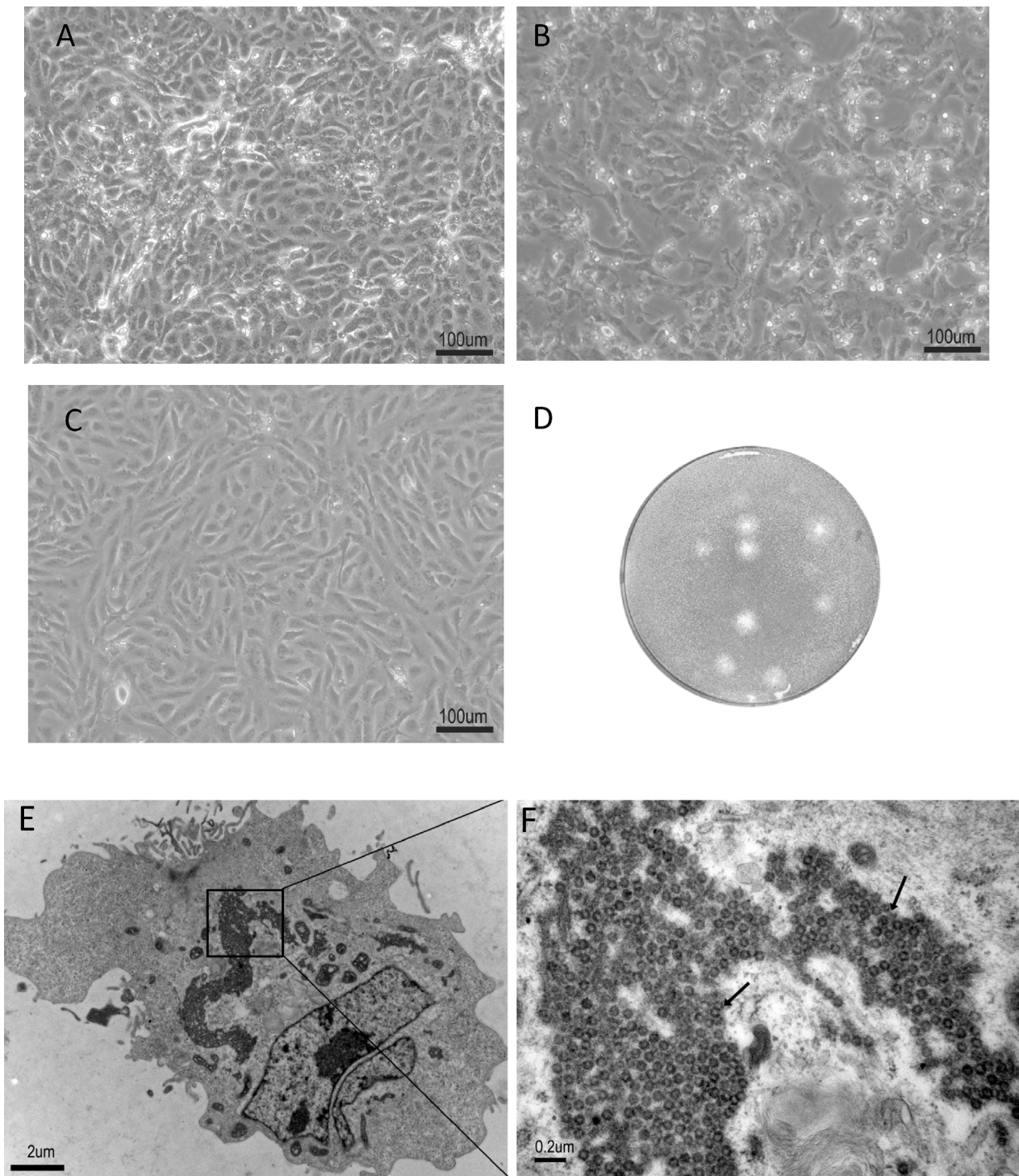
Fig. 1. Cytopathic effect (CPE), virus plaques and electron microscopy observation of the MRV-ZJ2013 in Vero cells. The infected cells were characterized by rounding and enlarging (indicated by arrows), then detachment. The infected cell monolayers were completely detached by 72–96 hpi. (A, Infected, hpi = 20 h; B, Infected, hpi = 72 h; C, Mock-infected, hpi = 72 h). Virus plaques, stained with crystal violet dye, were initially observed at 48 hpi (D). Electron microscopy of a negatively stained virus-infected Vero cells showed a single cells infected with MRV-ZJ2013 (E; hpi = 12). The virus packed closely in the cell cytoplasm (F).

such as PEDV, TGEV, PDCoV and RV initially, the purified viruses were subjected to the next generation sequencing. After quality control to remove reads shorter than 80 bases and duplicate reads, the sequencing data were assembled and compared with the GenBank database using the BLASTx algorithm. The results revealed the genomic identity of this virus to be MRV, and no other viral matches were detected (data not shown). An anti-σ1 rabbit polyclonal antibody was generated and used for IFA, with specific fluorescence observed in Vero cells infected with

MRV-ZJ2013, whereas staining with the pre-immune rabbit sera in virus-infected cells displayed no fluorescent signal (data not shown).

The *in vitro* growth kinetics of MRV-ZJ2013 indicated that infectious titers peaked at 36 hpi with a maximum of $10^{8.33}$ TCID$_{50}$/ml at an MOI = 0.01 (Fig. 2A). The virus completely lost infectivity under 254-nm UV irradiation for 20 min (Fig. 2A). The virus maintained high infectivity at 37 °C, but significantly lost infectivity at higher temperatures, with complete loss after 1 h at 100 °C (Fig. 2B).
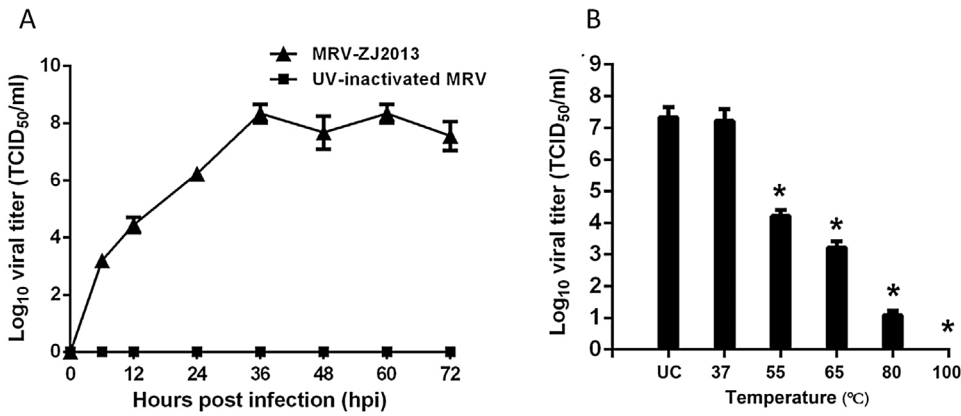
Fig. 2. (A) *In vitro* growth kinetics of MRV-ZJ2013. Confluent monolayers of Vero cells were infected in triplicate with MRV-ZJ2013 at an MOI = 0.01. At 6, 12, 24, 36, 48, 60, and 72 hpi, cells were harvested and virus titers (TCID$_{50}$) were determined in triplicate on Vero cells. UV-inactivated MRV was used as a negative control. The mean of the log-transformed titers are shown ± the standard error of the mean (SEM). (B) Inactivation of infectivity of MRV-ZJ2013 by heat treatment. Infectious virus titers (TCID$_{50}$) after incubation at different temperatures were compared with the untreated control (UC). Differences in the titer were evaluated by *t*-test with threshold of statistical significance indicated (*, $p < 0.05$).
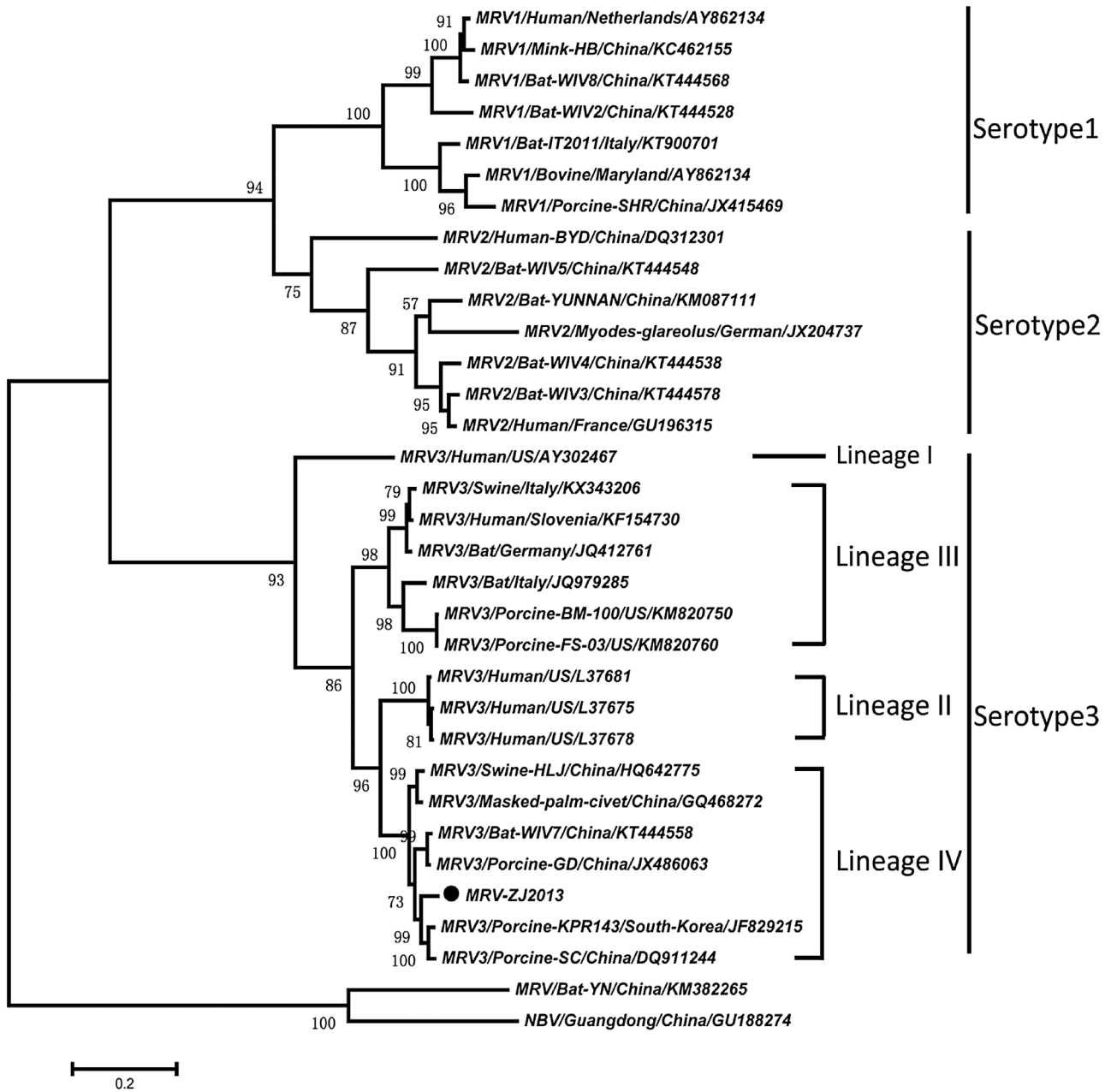

Fig. 3. Phylogenetic tree based on the S1 gene of MRV-ZJ2013. The phylogenetic tree was constructed by the maximum likelihood method using the Jukes-Cantor evolutionary model in MEGA5.2 software. Bootstrap values are indicated for each node from 1000 resamplings. The names of the strains, host species, places of isolation, GenBank accession numbers, and serotypes are shown. A solid circle indicates the MRV-ZJ2013 strain in this study.
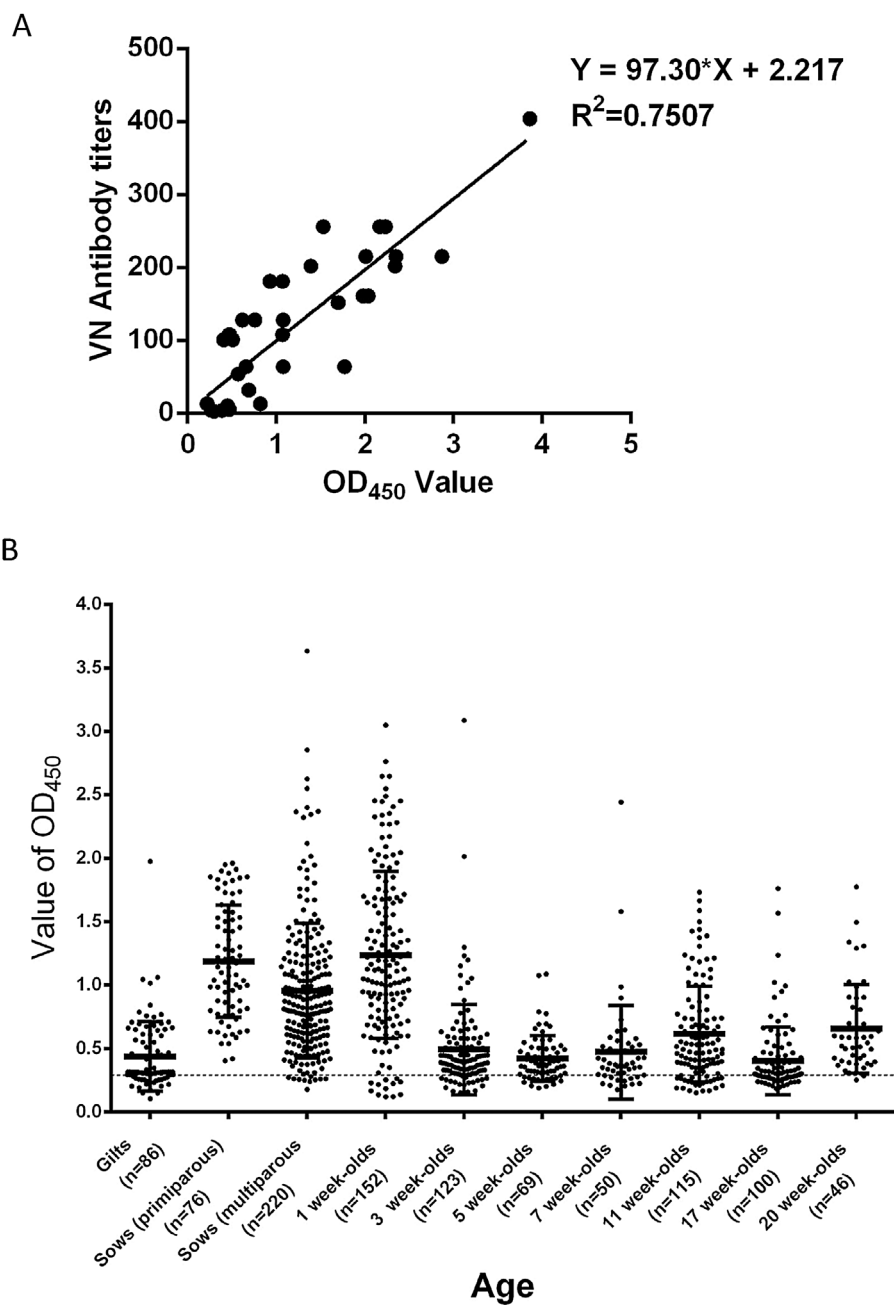
A

B

Fig. 4. Serologic profiles of anti-MRV IgG antibodies. (A) Scatter plots showing a correlation between the anti-σ1 IgG antibodies and the serum VN antibodies. (B) Distribution of σ1-based ELISA results (anti-MRV IgG antibodies) among serum samples. The samples were collected from sows and piglets with diarrhea at different ages. Positive cut-off value for the ELISA (0.32) is indicated by (−).

3.2. MRV-ZJ2013 is likely a reassortant MRV3 strain

As the full-length genome sequences with the 10 segments were not completely determined from the next generation sequencing, specific primers were designed to amplify all 10 viral segments by RT-PCR followed by molecular cloning and determination of the consensus sequences (data not shown). The complete genome sequence of MRV-ZJ2013 is available in GenBank under the accession numbers KY419120 to KY419129, corresponding to 10 genomic segments, respectively.

For each segment, the percentages of nucleotide and deduced aa sequence identity, relative to existing prototype strains and porcine MRV w analyzed (data not shown). Sequence analysis of MRV-ZJ2013 showed that while L1, M2 and S1 were identical to MRV3, the other seven segments were highly similar to MRV1. Putative aa sequence analysis showed similar results: λ1, μ1, μNS and σ1 (encoded by L1, M2, M3, and S1, respectively) were homologous to MRV3, while the

rest appear to originate from MRV1. Among the porcine orthoreoviruses, MRV-ZJ2013 had the lowest score of identity with those isolated from the USA.

To establish the evolutionary relationship between MRV-ZJ2013 and other known orthoreoviruses, phylogenetic trees were constructed based on the nucleotide sequences of each of the 10 segments. Based upon the S1 gene, MRV-ZJ2013 belongs to the lineage IV of MRV3 and is closely related to the other porcine MRV3 isolates from Asia (Fig. 3). Six segments are closely related to bat MRVs isolated in China: M1 and S3 likely originate from serotype 1 bat MRV; M3 and S2 likely from the serotype 2 bat MRV; and L2 and S4 likely from serotype 3 bat MRV (Supplemental Fig. S1). Other segments, such as L3, M2 and S1, have closest relationship with porcine MRV3 reported in Asia. However, the L1 gene of MRV-ZJ2013 is closely related to the sequence KT224504 derived from the mink MRV3 (Mink-SD) isolated in China (Supplemental Fig. S1). It's noted that the porcine MRV reported recently in the USA and Europe (Lelli et al., 2016; Thimmasandra Narayanappa et al.,
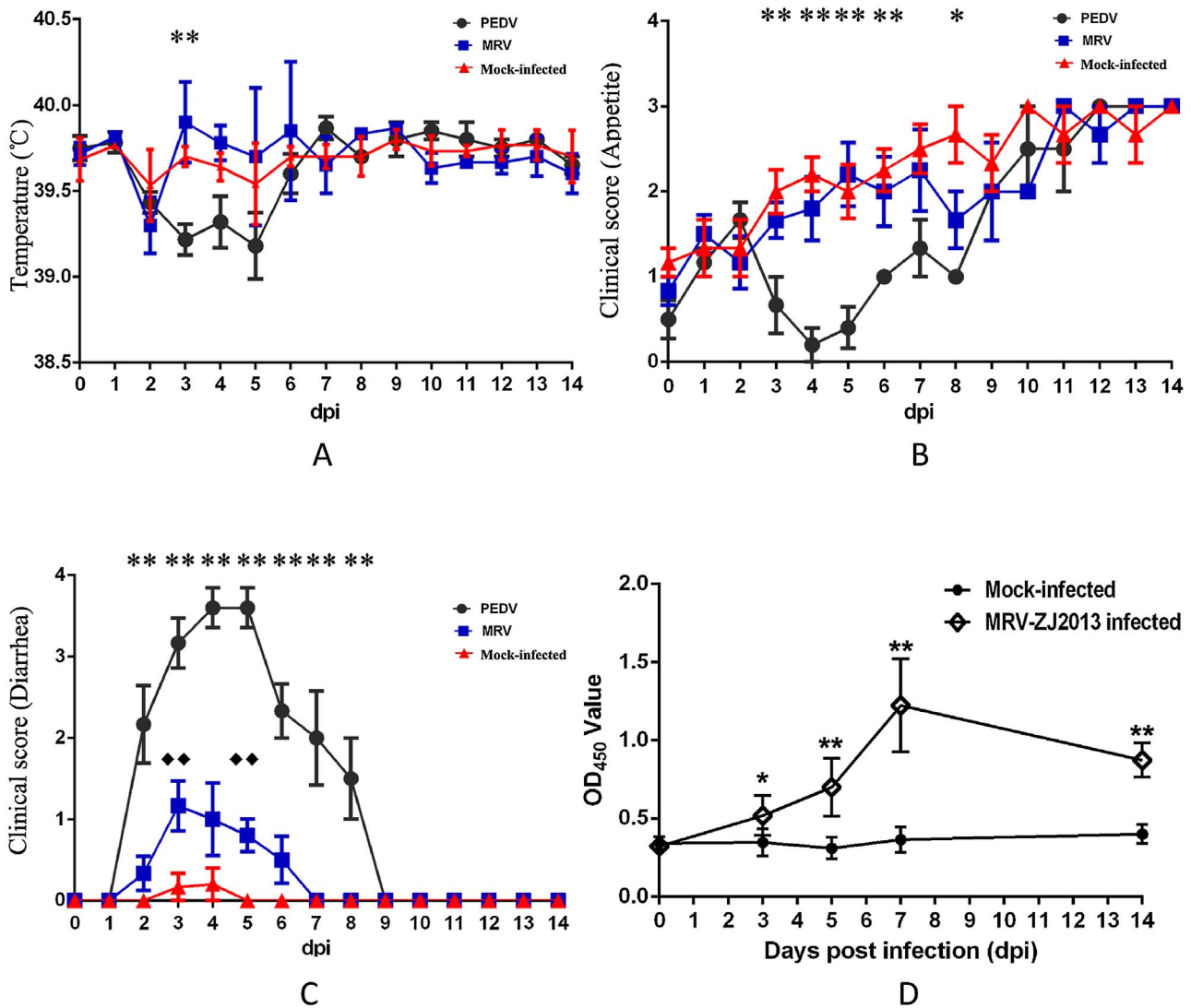
Fig. 5. Mean clinical score of piglets from 0 to 14 days post-infection (dpi). (A) Rectal temperature of the piglets. (B) The clinical scores of appetite: 1 – stopped feeding, 2 – inappetence, 3 – inactivity and 4 – normal. (C) The clinical score of diarrhea: 1 – normal, 2 – soft, 3 – diarrhea, and 4 – watery. Differences in the clinical scores were evaluated by ANOVA. Asterisks indicate significant difference between the PEDV challenged group and the DMEM control group, $**p < 0.01, *p < 0.05$. ♦ indicates significant differences between the MRV-ZJ2013 challenged group and the DMEM control group, $♦♦ p < 0.01, ♦ p < 0.05$. (D) Comparison of OD450 nm values of serum samples collected from the MRV-ZJ2013 challenged group and the mock-infected group in the pathogenesis study. Differences in the OD450 nm values of serum samples from different groups were evaluated by t-test. Asterisks indicate significant difference between the two groups, $**p < 0.01, *p < 0.05$.

2015), which causes clinical diarrhea and acute gastroenteritis, has completely different origins than MRV-ZJ2013. The close genetic similarity of different segments of MRV-ZJ2013 to those of the respective bat, mink and porcine MRVs might reflect recent cross-species transmissions in pig herds, and suggesting the hypothesis that MRV-ZJ2013 is likely a reassortant MRV3 strain.

### 3.3. A high seroprevalence of MRV in diarrheic pigs with a wide range of ages

Preliminary neutralization tests using selected porcine sera demonstrated a widespread distribution of the MRV3 VN antibody, with about two-third of them having a titer over than 100 (Fig. 4A). The anti-σ1 IgG antibody levels among these samples were also determined by indirect ELISA. A good linear relationship between the anti-σ1 antibodies and VN antibodies was observed (Spearman's rank correlation coefficient of 0.91; P < 0.01), demonstrating the correlation between them (Fig. 4A). We thus apply the σ1-based ELISA to the subsequent seroepidemiological survey of MRV3.

A total of 1037 serum samples collected from diarrheic pigs were tested (Fig. 4B). The serum samples were collected from a wide range of ages, from suckling piglets to sows. A high positive incidence was observed in the sows (94–100%), 1-week-old piglets (94%) and the 20-week-old fattening pigs (91%). However, the highest OD values were observed in the sows and the one-week-old piglets. Newborn piglets (represented by the 1 week-old piglets) receive maternal IgG antibodies from sows via the colostrum, though the level of the antibodies decline quickly, and 3 week-olds weaning piglets must begin to develop their own immunity to the virus. Among the sows, gilts had the lowest level of antibodies similar to the weaned and growing pigs, supposedly increasing with parity (Fig. 4B). Overall, the data revealed a high seroprevalence (77%) of MRV3 from 1037 serum samples collected from diarrheic pigs of different ages in seven provinces of east China.

3.4. MRV-ZJ2013 had low pathogenesis in newborn piglets

It has been shown that experimental PEDV infection caused clinical symptoms in neonatal piglets (Zhang et al., 2015). In order to
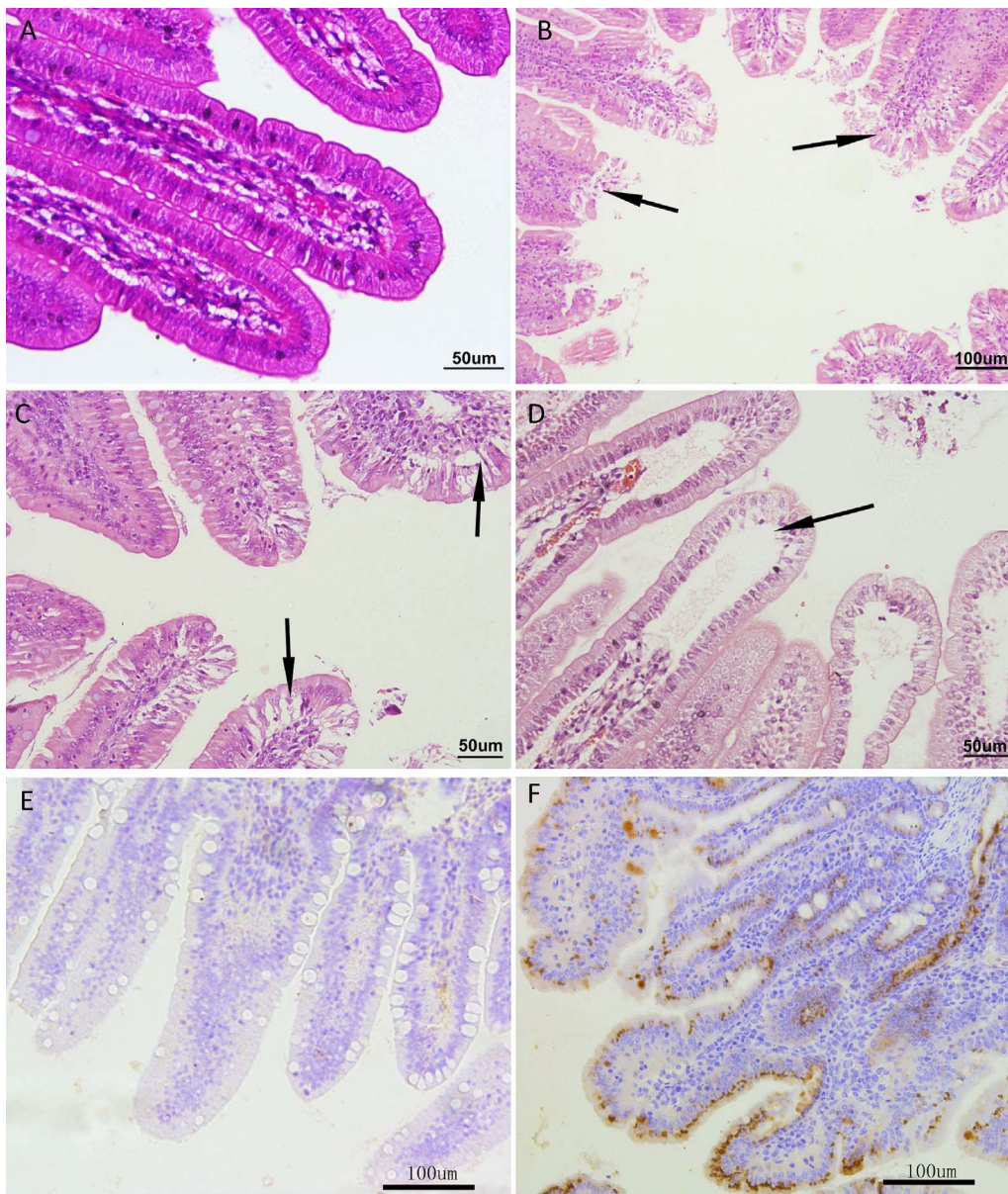
Fig. 6. Representative histological examinations (A–D) and IHC (E and F) of the mid-jejunum collected from piglets of the pathogenesis experiment. A, Mock-infected; B, PEDV challenged; C and D, MRV-ZJ2013 challenged; E, Mock-infected; F, Infected with MRV-ZJ2013. Arrows indicate capillaries, capillary lymphatics, and the central lacteals of the intestinal villous gradually atrophied and diminished.

determine whether the reassortant MRV-ZJ2013 strain is pathogenic similar to the virulent PEDV, a comparative pathogenesis study was performed. Three groups with six piglets each were inoculated with DMEM, PEDV and MRV-ZJ2013, respectively. Prior to the study, fecal samples from all 18 newborn piglets were tested negative for PEDV, PDCoV, TGEV, RV, and MRV by RT-PCR.

There was no significant variation in rectal temperature between the MRV3-infected group (group C) and the mock-infected group A throughout the study (Fig. 5A). In contrast, pigs in the PEDV-infected group (group B) reduced rectal temperature significantly at 3 dpi (Fig. 5A). Accordingly, there is evidence to support that MRV3-infected pigs had mild symptoms such as poor appetite and soft feces between 3 and 5 dpi, but lacked vomiting and diarrhea, which appeared in piglets of the PEDV-infected group (Fig. 5B and C). Fecal virus shedding was detected only in three piglets, each for one- or two-day post-infection. Discernable salient features of viral infection were apparent in the PEDV challenged group: diarrhea was observed in some piglets at 2 dpi and all piglets had diarrhea by 4 dpi. Detection of the anti-σ1 antibodies by indirect ELISA showed that the antibodies level increased at 3 dpi, with the peak level reached at 7 dpi and then decreased, though they

remained detectable at 14 dpi (Fig. 5D).

Upon histopathological analysis, microscopic lesions of the duodenum were detected in the PEDV-infected group including: necrosis of scattered enterocytes; enterocyte attenuation; and sloughing of degenerate and necrotic enterocytes, whereas no intestinal lesions were observed in control pigs (Fig. 6A and B). The pathologic changes in the intestine of MRV-ZJ2013 challenged group were milder than those of the PEDV group, showing gradual atrophy and diminishing of capillaries, capillary lymphatics and central lacteals of the intestinal villous (Fig. 6C and D). Immunohistochemistry showed that MRV antigen was not detected in the duodenum at 3 dpi, but intense specific immunohistochemical signals could be observed at 5 dpi, by using the rabbit anti-σ1 antibody (Fig. 6E and F). Antigens were detected in the entire villous epithelial cells in the mid-jejunum of MRV-ZJ2013 inoculated piglets. It was possible that the piglet euthanized at dpi 3 had weak infection signal lower than the IHC detection limit due to individual differences. According to these results, we consider MRV-ZJ2013 to be a low, if any, pathogenic strain, which can elicit an immune response without significant clinical signs.
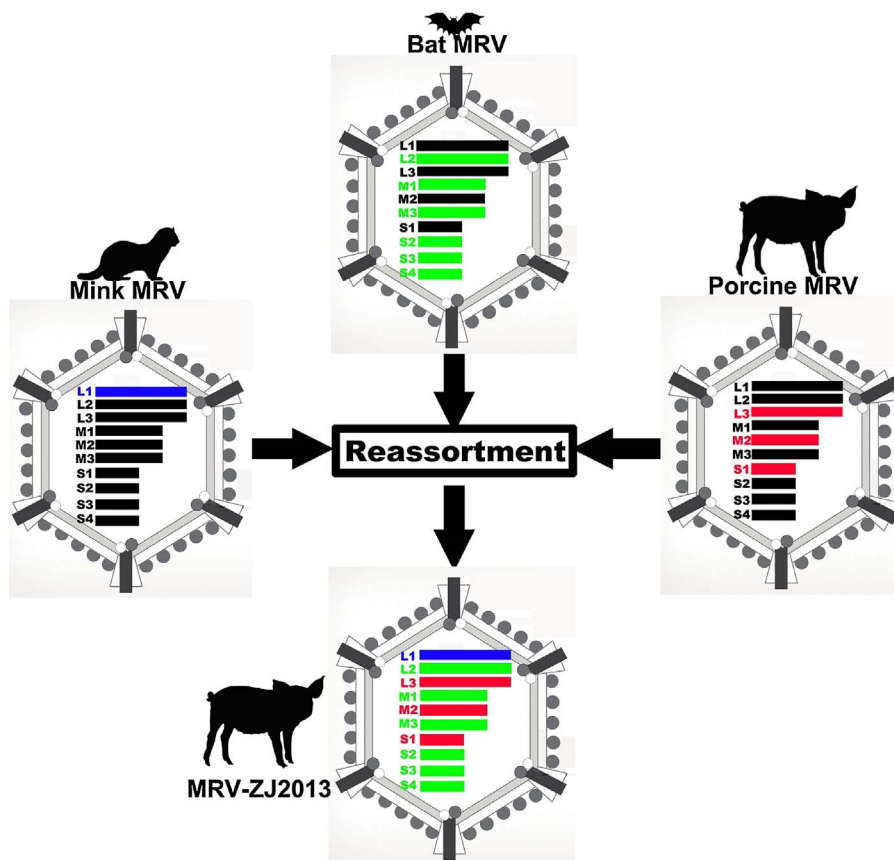
4. Discussion

Frequent outbreaks of diarrhea cause substantial economic losses for the swine industry, with PEDV, TGEV, PDCoV, RV, EV, and some bacteria as the primary agents. However, some newly emerging pathogens have been reported in the past decade. We have isolated a porcine MRV3 from the feces of diarrheic pigs in Zhejiang province in China (Fig. 1), and ELISA has shown that anti-MRV3 σ1 IgG antibody is present in the majority of diarrheic pigs at different ages (Fig. 4B). As the serum samples in our study were collected from pigs at a wide range of ages, the results give a good overview of viral prevalence. The current study shows that sows and 1-week-old piglets had a high positive rate as well as high levels of anti-MRV IgG. The mean OD450 of primi- and multiparous sows was higher than the gilts, indicating that anti-MRV3 IgG levels increase over time (Fig. 4B). It also indicates that piglets had already been infected at 1 week-of-age, and the sows are likely viral sources. Maternal antibody levels decreased gradually over time, disappearing around the weaning age (Pignatelli et al., 2010), after which the piglets developed their own immune response upon viral exposure. The study shows that anti-MRV IgG exists at all ages, suggesting that there may be a continuous spread of virus within the farms. For detection of the MRV3 RNA, our preliminary data using limiting samples revealed that porcine MRV3 was present in at least 24 of 208 (11.5% positive after first round, and 66% positive after nested round) diarrheic porcine fecal samples obtained from different provinces by the nested RT-PCR assay described above targeting the L1 gene (unpublished data). It has also been reported that porcine MRV RNA was detected in 19% of diarrheic fecal samples collected from 78 pig farms in South Korea (Kwon et al., 2012b). The prevalence of MRV3 in North American pigs has recently been investigated with a real-time RT-PCR designed to target the S1 gene of MRV3, showing that MRV3 RNA was detected in 5.7% (16/277) of the fecal samples from 19.5% (8/41) of the farms (Xiao et al., 2016). These results indicate that many

herds all over the world are becoming exposed to this virus.

The dsRNA genome of MRV contains 10 discrete segments, which are prone to various types of alterations including intragenic rearrangement and reassortment when different strains concurrently infect a single host cell, both in laboratory and natural conditions (Lian et al., 2013; Mertens, 2004). We found the genome of MRV-ZJ2013 to be a likely reassortment of segments from mink, bat, and pig MRVs (Fig. 7), similar to previously reported phenomena (Yang et al., 2015). Moreover, an RDP4 (Recombination Detection Program) analysis on the concatenated ten segments showed that MRV-ZJ2013 was derived from several cross-over events with well-supported breakpoints detected near the boundaries of the segments L1-L2, M1, M2, M3 and S1 in the concatenated genome (data not shown), which was in lines with the phylogenetic analyses. We speculate that bats are the primary hosts for genetic exchange among different MRV strains under natural conditions, as they possess certain ideal characteristics such as long life span, diversity, wide distribution, and they carry a number of zoonotic viruses from many different virus families (Smith and Wang, 2013). The last decade has experienced a surge in reports of MRVs of bat origin that have a significant impact on human and animal health (Hu et al., 2014; Kohl et al., 2012; Steyer et al., 2013b; Wang et al., 2015a). Moreover, the genome of MRV-ZJ2013 shows reassortment among different bat MRV serotypes: M1 and S3 seem to originate from serotype 1 bat MRV; M3 and S2 seem to come from serotype 2 bat MRV; and L2 and S4 come from serotype 3 bat MRV. Reassortment among different strains or recombination between different species may affect viral pathogenesis and zoonotic potential. Indeed, our previous study on emergent PEDV also identified a possible recombination event between PEDV and bat coronavirus in pigs (Huang et al., 2013). Whether the virulence of the reassorted MRV-ZJ2013 may have been relatively decreased will be determined in the future.

The pathogenesis study showed that MRV-ZJ2013 from a diarrheic piglet was more likely non-pathogenic. This was probably due to mixed

infections of MRV with the other viruses associated with diarrhea such as PEDV in the original sample. Additionally, piglets could develop an immune response without experiencing severe clinical symptoms including vomiting and diarrhea (Fig. 5). Phylogenetic trees based on the S1 gene show that MRV-ZJ2013 and porcine MRVs isolated in the USA and Italy may have different origins. The strains isolated in the USA and Italy belonged to the lineage III. However, MRV-ZJ2013 and other Asia strains fall into the lineage IV (Fig. 3). The σ1 protein is responsible for cell attachment, specifying tissue tropism, and hemagglutination. It consists of 453 aa with three discrete domains: the tail (310–455 aa), body (170–309 aa), and head (1–160 aa) (Reiter et al., 2011). Serotype 3 (T3) reoviruses use JAM-A and α-linked sialic acids as receptors; structural and functional analyses show that the σ1 head engages JAM-A while the σ1 body binds to α-linked sialic acid (Reiter et al., 2011). Antigenic analysis shows that porcine MRVs isolated in the USA and Europe share a similar antigenic index, whereas Asian porcine MRV strains such as MRV-ZJ2013 and isolates from the Sichuan province of China and South Korea are very different (Supplemental Fig. S2). Compared to the σ1 protein of MRV-ZJ2013, there are many unique aa substitutions in the head, body, and tail domains of the σ1 protein in porcine MRVs of US origin, with an associated difference in antigenic index that may contribute to variation in MRV pathogenesis (Supplemental Fig. S2).

This study, along with a previous one (Yang et al., 2015), shows evidence of possible zoonotic transmission events among viral strains from different host. An antigenically novel variant may be produced during reassortment that is not recognized by the host immune system, creating higher-fitness variants that can adapt to new hosts. Understanding the consequences of viral reassortment is important, as it may lead to newly emerging pandemic reoviruses with dire consequences for humans and animals (Mackenzie and Jeggo, 2013; McDonald et al., 2016).

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetmic.2017.07.021.

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