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Recent Outbreaks and emergence of mutants of Porcine Epidemic Diarrhea Viruses (PEDV) in Korea

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Porcine epidemic diarrhoea virus (PEDV) and porcine deltacoronavirus (PDCoV), which belong to the genera alphacoronavirus and deltacoronavirus, respectively. PEDV was first reported in South Korea in 1992 (Kweon *et al.*, 1993), with the occurrence of an outbreak, and has since circulated with considerable genetic diversity (Song and Park, 2012). During 2013, PED outbreaks reoccurred in South Korea; however, the emerging PEDVs in these outbreaks were not variants of previous Korean isolates or attenuated vaccine strains (Song and Park, 2012). Although vaccines (DR13, SM98 strains) which have been developed and used in commercial swine farms for prevention of this disease, efficacy is now doubtful (Chung *et al.*, 2015; Lee and Lee, 2014; Tian *et al.*, 2013). Besides, it has severely attacked the United States in April 2013, and led to significant economic losses in pig industry even they have adopted way of import restriction (Song and Park, 2012). This report will further describe the epidemiological situation of PEDV infection based on information reported in the last 4 years (2012–2015), including the spread of PEDV to the South Korea.

In contrast, PDCoV was only recently

described. Detection of PDCoV has currently only been reported in Hong Kong, the USA and Canada, South Korea, and China (Wang *et al.*, 2014a; Wang *et al.*, 2014b; Lee and Lee, 2014). More recently, a novel emerging PDCoV was demonstrated to be enteropathogenic, causing severe diarrhea similar with those of PEDV and TGEV infections, and mild interstitial pneumonia (Jung *et al.*, 2015). We will further report the presence and genetic characterization both PEDV and PDCoV from cases that showed symptoms of diarrhea in Korean swine farms.

1. Epidemiological situation regarding PEDV and PDCoV

1.1. Collection of information

Among samples requested for diagnosis of PED, prevalence and pattern were verified and compared between 2012–2013 and 2014–2015 periods, which were divided according to the point of unexpected blow of PED in the United States. There were 607 fecal samples (63 farms) from October 2012 to December 2013, and 223 fecal samples (36 farms) from January 2014 to

Table 1. Numbers of tested, positive samples and prevalence rates in respective age groups according to study periods; the year of 2012–2013 and the year of 2014–2015

The year of 2012–2013	Suckling	Weaned	Growers	Finishers	Gilts	Sows	Total
Number of samples	440	88	22	12	12	33	607
Positive samples	3	2	0	0	0	0	5
%	0.68%	2.27%	0%	0%	0%	0%	0.82%
The year of 2014–2015	Suckling	Weaned	Growers	Finishers	Gilts	Sows	Total
Number of samples	92	40	54	20	4	13	223
Positive samples	25	8	6	4	1	2	46
%	27.17%	20%	11.11%	20%	25%	15.38%	20.63%

March 2015. All these samples were randomly collected from commercial swine farms in nine provinces of South Korea. Age groups ranged from suckling to sows. Also, for screening of PDCoV, feces samples of pigs that showed signs of diarrhea (n = 681) were collected from 59 commercial pig farms January 2013 through March 2015.

1.2. Occurrence of PEDV and PDCoV

The result (Table 1) indicates the presence of PEDV in porcine fecal samples according to study periods. The difference of PEDV detection between the periods was clearly presented. Total detection rates of each period were around 0.82% (5 positives/607 total samples) and 20.63% (46 positives/ 223 samples). According to age groups, during 2012–2013, 3 positives in suckling and 2 positives in weaned pigs were detected respectively. There was no positive sample in gilt and sow groups. No other age groups in that time were infected. On the other hand, during 2014–2015, all age groups were infected with PEDV, showing the highest detection rate in suckling piglets. In 2012–2013, both Gyeonggi and Gyeongbuk provinces were shown to be positive. However, in 2014–2015, all provinces except Gangwon and Jeju were shown to be positive.

The PDCoV screening results by RT-PCR with 581 samples showed that until 2014, all of tests were shown to be negative. It was on March 2015 that, the first PDCoV positive samples were detected in a 600- scale sow farm (SL farm) in

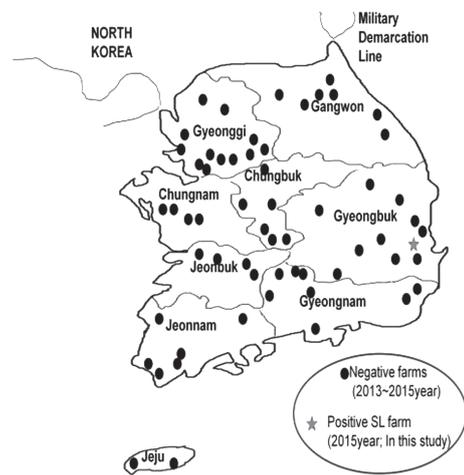


Fig. 1. The distribution of sampling farms in South Korea. The locations of farms were indicated and marked as ● for PDCoV negative farms, and as ★ for PDCoV positive farms.

Gyeongbuk province (Fig. 1). This farm was reported to be infected by PEDV in 2014, with severe diarrhea of 100% mortality in piglets. In early 2015, it was observed that up to 20% pigs of all ages had diarrhea and 10% of the pigs died. The diagnosis of porcine enteric viruses revealed the dual infection of PDCoV and PEDV, while TGEV, group A rotavirus and Kobuvirus were not detected.

2. Isolation of PEDV

2.1. Description

Attempts to isolate the field strains of PEDV on Vero cell lines followed a previously described

protocol with modifications (Chen, Q *et al.*, 2014). An overnight monolayer of Vero cells (80%–100% confluence) was washed twice with $1\times$ phosphate-buffered saline before homogenized samples ($0.02\ \mu\text{m}$ filtered) were inoculated with 10% suspension. After 30 min absorption at 37°C with 5% CO_2 , maintenance medium (Dulbecco's Modified Eagle Medium supplemented with trypsin [$10\ \mu\text{g}/\text{mL}$], yeast extract (0.04%), tryptose phosphate broth (0.6%), and Antibiotic-Antimycotic 100x ($4\ \mu\text{l}/\text{mL}$; Gibco, Thermo Fisher Scientific, Grand Island, NY, USA) were added at a ratio of 1 : 10. The inoculated cells were cultured for 3–4 days at 37°C in 5% CO_2 atmosphere and were blindly passaged 5 times. Although there were PEDV positive collected samples ($n = 30$) confirmed by RT-PCR, only one field strains of PEDV (BM1) were successfully adapted to grow on Vero cells for 10 serial passages.

2.2. PEDV Titers and IFA

The BM1 PEDV field isolate induced cytopathic effects of rounded shape (Fig. 2, panel A) within 48 hours at passage 10. The presence of PEDV in the cell culture was confirmed by immunofluorescence assay (VDPPro PEDV FA Reagent kit, MEDIAN Diagnostics, Gangwon-do, South Korea), which showed the specific fluorescence signal (Fig. 2, panel B). In addition to evidence by microscopic observation, real-time reverse transcription PCR showed that the quantity of viral RNA increased incrementally as the number of passages increased: from 30,325 copies/ μL (cycle threshold 16.11) at passage 2 to 418,000 copies/ μL (cycle threshold 13.77) at passage 10. Infective titers of the BM1 isolate increased from $10^{4.7}$ 50% tissue culture infectious doses/mL at passage 2 to $10^{7.9}$ 50% tissue culture infectious doses/mL at passage 10 (Table 2).

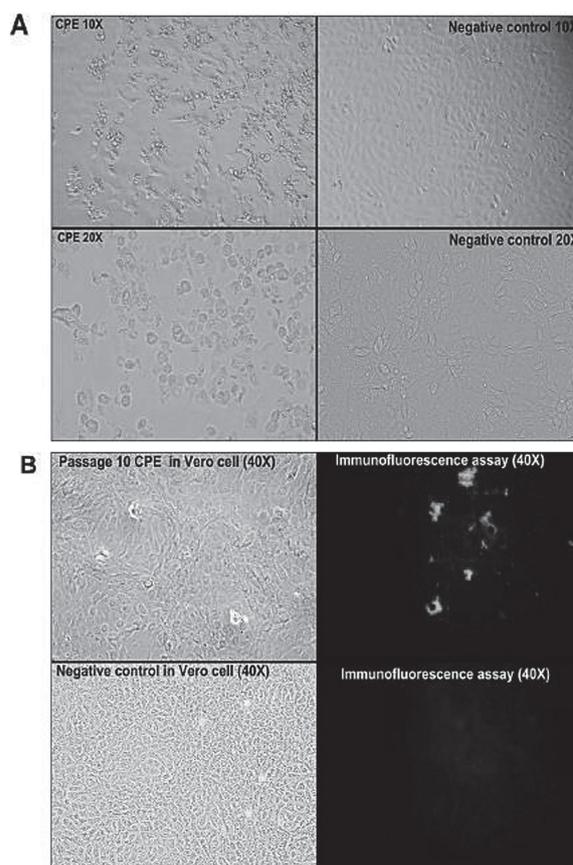


Fig. 2. Results of microscopic examination and specific IFA staining of Vero cells infected by BM1 isolate of porcine epidemic diarrhea virus at passage level 10. A) Cytopathic effects and B) Immunofluorescence assay.

3. Genetic characterization PEDV and PDCoV

3.1. Differences between South Korea and American PEDV isolate

The complete S gene of BM1 (GenBank accession no. KP861982) was sequenced for genetic characterization; the gene was 4,161-nt long and encoded 1,386 aa. The spike protein of the BM1 isolate showed substitutions at neutralizing SS6 epitope from LQDGQVKI (Lee DK *et al.*, 2011) to SQSGQVKI but identity at the SS2 and 2C10 neutralizing epitopes (Lee DK *et al.*, 2010). The genetic relationship of the BM1 isolate with other PEDVs in the world was inferred from a codon-based alignment of sequences ($n = 409$) of the complete S gene. The maximum-likelihood phylogenetic tree was constructed by using the

Table 2. Titration of BM1 PEDV isolate from passage 1 to passage 10, South Korea, 2013–2014

BM1 sample parameter	Intestine†	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
Cytopathic effect	ND	ND	+	+	+	+	+	+	+	+	+
RT-PCR	+	+	+	+	+	+	+	+	+	+	+
Real-time (copies/ μ l; C_T value)	1,412; 17.96	1,905; 17.91	30,325; 16.11	21,633; 16.42	24,727; 16.3	7,287; 17.39	16,565; 16.77	33,905; 16.02	80,579; 15.24	283,000; 13.98	418,000; 13.77
Infectious titer (\log_{10} TCID ₅₀ /ml)	ND	ND	4.7	4.2	5.2	2.7	5.2	5.7	6.2	7.2	7.9

*ND, not determined; P, passage; PEDV, porcine epidemic diarrhea virus; RT-PCR, reverse transcription PCR; +, positive. †0.02 μ m filtered of 10% intestine homogenized suspension.

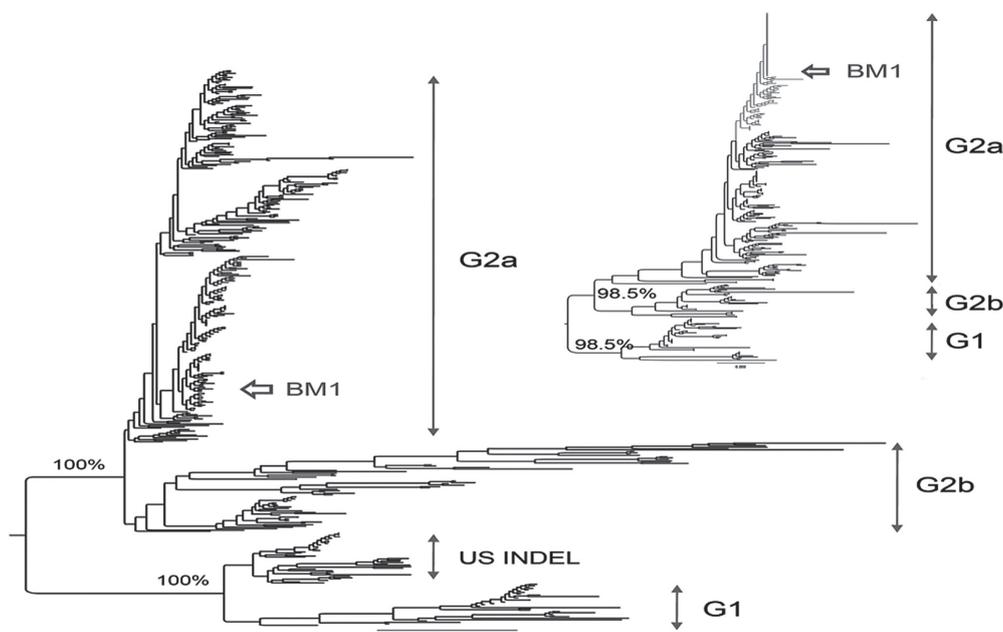


Fig. 3. Maximum-likelihood phylogenetic tree of porcine epidemic diarrhea virus from piglet, South Korea, 2013–2014, constructed on the basis of codon alignment of complete S genes. Inset shows a phylogenetic tree inferred from the complete N genes. Genogroups are shown to the right of each tree. US INDEL is a prototype strain of porcine epidemic diarrhea virus that has insertions and deletions (INDELs) in the spike gene. Scale bars indicate nucleotide substitutions per site.

FastTree program, with the general time reversible nucleotide substitution model. The phylogeny constructed on the basis of the complete S gene showed that the BM1 isolate belongs to subgroup 2a, genogroup 2 of PEDV. This isolate clustered closely with emergent PEDV strains in the United, showing 99.2%–99.7% identity with PEDVs of North American strains. This observation was repeated by the phylogenetic inference of the complete N. The branching pattern clearly showed that BM1 is genetically less related (92.9–93.4% identity) to

the live vaccine strains that are derived from genogroup 1 and used currently to prevent PEDV infections in South Korea (Fig. 3).

3.2. Genetic characterization of PDCoV as an emerging disease

For the genetic characterization, the maximum likelihood phylogenetic trees that was reconstructed based on the S and N genes (Fig. 4A, B) showed a clear separation between Chinese and US strains of PDCoV, and the result is similar with the previous studies (Wang *et al.*,

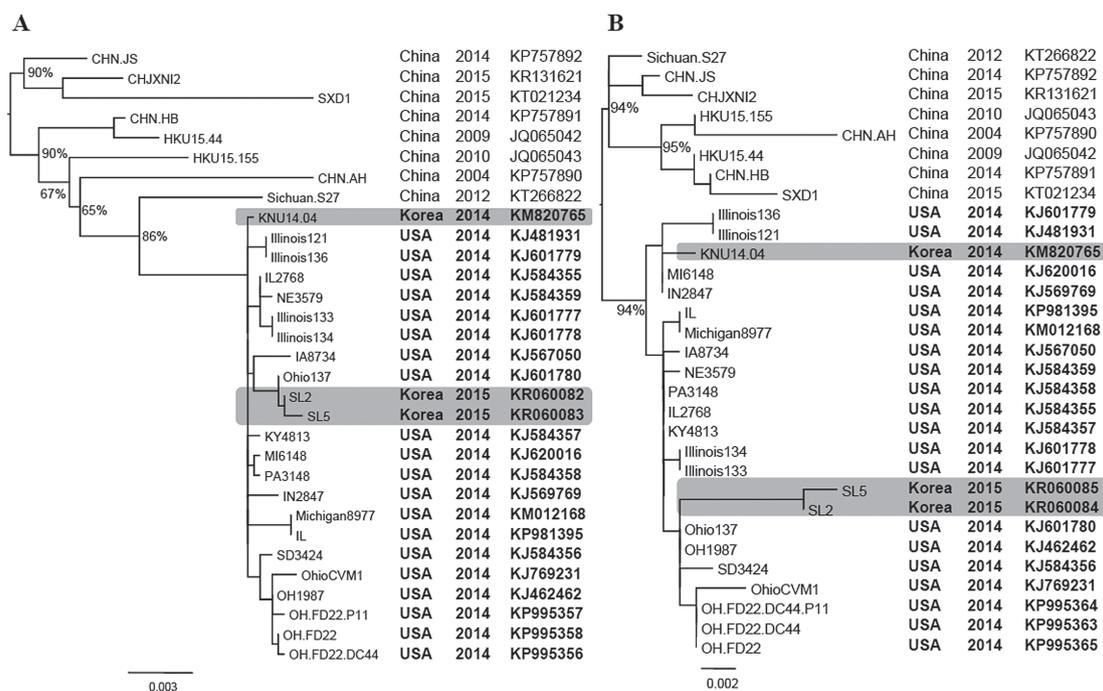


Fig. 4. Maximum likelihood phylogeny of PDCoVs based on the spike protein coding gene (A) and the nucleocapsid protein coding gene (B). The numbers at the nodes of the phylogenies denote the bootstrap values to which they belong (for clarity, labels of some terminal nodes were omitted). The phylogenetic trees showed that Korean PDCoV isolates in 2014 (KNU14.04) and in 2015 (SL2, SL5) were grouped within US PDCoV cluster, but they were located at different branches (highlights).

2015, Marthaler *et al.*, 2014). Among them, Korean strains of PDCoV isolated in 2014 (KNU14.04) and in 2015 (SL2 and SL5) were grouped within US PDCoV cluster, however, they were located at different branches (highlights, Fig. 4A, B). Based on the S gene, the inferred ancestral amino acid changes along the nodes of the phylogeny (Fig. 5A) showed that the branches leading to Korean PDCoV isolates in 2014 and in 2015 shared 1 back substitution (node 40: Q106L, node 37: L106Q) and 4 unique substitutions (node 39: S697A, node 38: V550A, I669L and node 37: I1014V). However, the branch that leads to 2015 isolates (SL2 and SL5), had further 2 mutations which were located near the tip of the phylogeny (node 59: I110V, T582A). Based on the N gene, it was observed that only amino acid mutations (6 changes) was near the tip of the phylogeny, on the node leading to SL2 and SL5 (Fig. 5B). At present, the significance of these substitutions is almost obscured. Of the all, the

phylogenetic analyses suggested that the PDCoVs strains (SL2, SL5) detected in early 2015 are different with the previously emerged virus (KNU14.04).

4. Emergence of Variant PEDV and development of MERS-CoV rapid diagnostic kit

Coronaviruses may infect many different animals and cause them to have respiratory, gastrointestinal, liver, and neurologic diseases. Most of these coronaviruses usually infect only one animal species or, at most, a small number of closely related species. However, some coronaviruses, like the one that caused SARS and MERS can infect people and animals. In field of veterinary virology, coronaviruses have caused damage to livestock industry continuously. Porcine epidemic diarrhea virus (PEDV), an Alphacoronavirus in the family Coronaviridae,

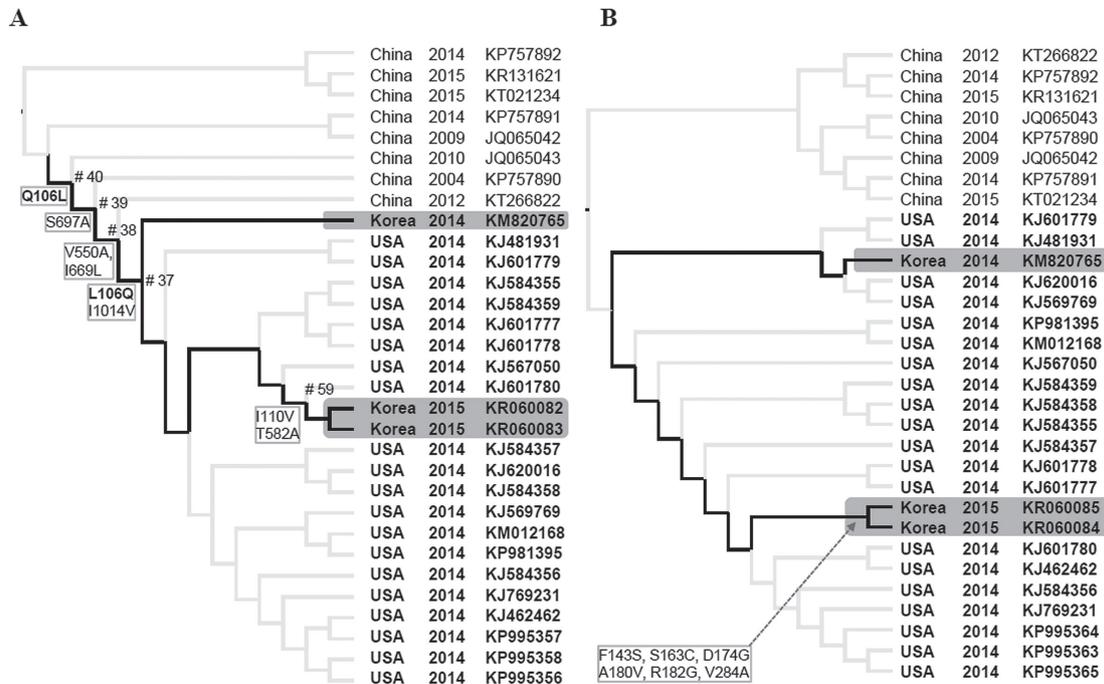


Fig. 5. The maximum likelihood trees based on S-gene (A) and N-gene (B) with reconstructed non-synonymous substitutions mapped to the nodes (#) of the phylogeny. For clarity, only branches leading to Korean PDCoV isolates were highlighted. It was observed that the branch which leads to 2015 isolates (SL2, SL5) accumulated further mutations compared to the branch which lead to 2014 isolate (KNU14.04).

causes acute diarrhea, vomiting, dehydration, and high mortality rates in neonatal piglets. PEDV can also cause diarrhea, agalactia, and abnormal reproductive cycles in pregnant sows. Although PEDV was first identified in Europe, it has resulted in significant economic losses in many Asian swine-raising countries, including Korea, China, Japan, Vietnam, and the Philippines. However, from April 2013 to the present, major outbreaks of PEDV have been reported in the USA, Canada, and Mexico. Moreover, intercontinental transmission of PEDV has increased mortality rates in seronegative neonatal piglets, resulting in 10% loss of the US pig population. The emergence and re-emergence of PEDV indicates that the virus is able to evade current vaccine strategies. Continuous emergence of multiple mutant strains from several regions has aggravated PED endemic conditions and highlighted the need for new vaccines based on the current circulating PEDV.

Coronavirus of Middle East was also

identified in animals, mainly in camels, and it infected people causing a respiratory syndrome, MERS (Middle East Respiratory Syndrome), in several countries. The MERS-CoV spread to other countries and caused grave damage to South Korea recently. To prevent new emerging zoonosis in advance, the development of diagnosis is strongly emphasized. We present here a rapid immunochromatographic assay for the detection of Middle East respiratory syndrome coronavirus (MERS-CoV) antigen in the nasal swabs of dromedary camels. The assay is based on the detection of MERS-CoV nucleocapsid protein in a short time frame using highly selective monoclonal antibodies at room temperature. The relative sensitivity and specificity of the assay were found to be 93.90% and 100%, respectively, compared to that of the UpE and open reading frame 1A (Orf1A) real-time reverse transcriptase PCR (RT-PCR). The results suggest that the assay developed here is a useful tool for the rapid diagnosis and epidemiological surveillance of

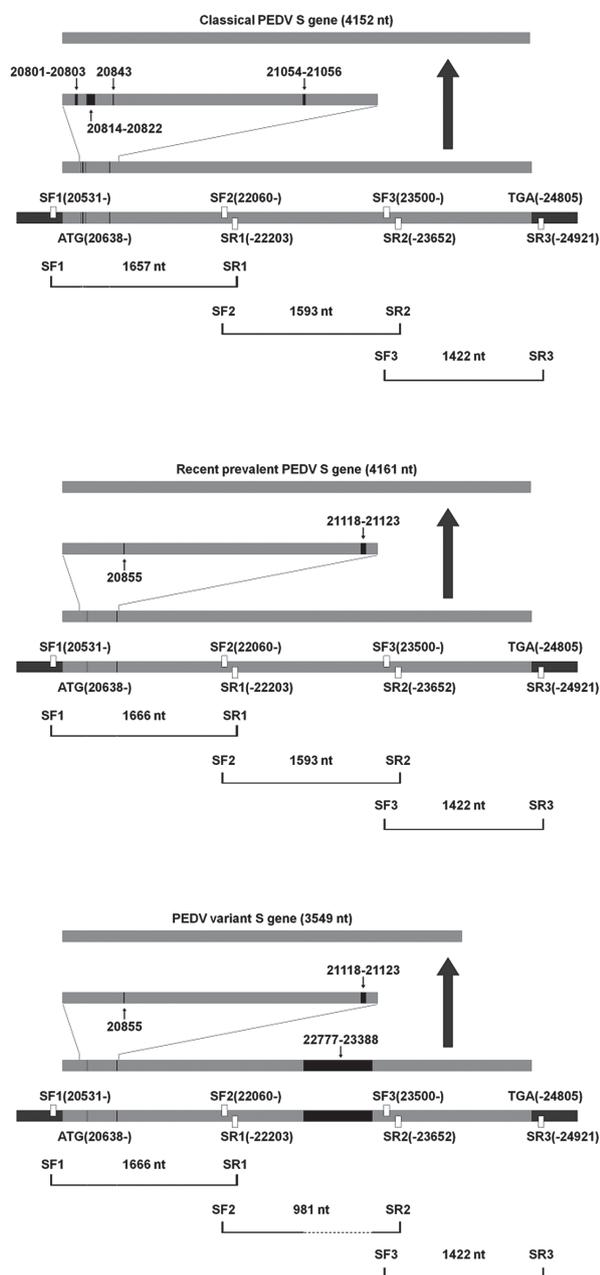


Fig. 6. Schematic diagram of the S genes of classical PEDV, recent prevalent PEDV and PEDV variant. cDNA clones for the entire S gene of classical PEDV, recent prevalent PEDV and PEDV variant were constructed by RT-PCR using pairs of sense (SF) and antisense (SR) primers: diagrammatic representation of the S gene (red rectangle) of viral RNA (blue rectangle) show primer-binding sites (small open rectangle). Nucleotide deletions (black rectangle) are indicated by arrow and their location are labeled above the arrow as nucleotide numbers. Nucleotide numbers shown in diagram correspond to those of the classical strain, CV777 (GenBank accession number AF353511) (Park *et al.*, 2015, EID).

MERS-CoV infection in dromedary camels.

5. Conclusions

PEDV and PDCoV are a rapidly evolving area of knowledge. This scientific report reflects current understanding at South Korea situation.

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