Detection and Genetic Characterization of Porcine Epidemic Diarrhea Virus in South Korea

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ABSTRACT

This study applied molecular-based methods to investigate emerging patterns of the recent porcine epidemic diarrhea virus in Korean domestic swine farms from 2016 to 2017. In our study, the finisher group showed the highest positive rate. By Sanger sequencing, this study recovered a complete genome of 28,005 bases long of NB1 strain from a severely PED affected farm. By application of Bayesian phylogeographical analysis, the complete genome of the virus (NB1 strain) showed that it is clustered with PEDV Korean strains, which differentiated from the USA that belongs to geno group 2a. This study also successfully reconstructed diffusion pathways of PEDV into Korea. Interestingly, though grouped within US prototype- like PEDVs, NB1 strain directly originated from a local lineage.

INTRODUCTION

Porcine epidemic diarrhea (PED) is an acute contagious diarrhea disease caused by porcine epidemic diarrhea virus (PEDV) in pigs.[6,17] The virus belongs to genus Alphacoronavirus, family Coronaviridae, which includes other genera, Beta-, Gamma-, and Deltacoronavirus, and has positive-sense single stranded RNA genome with envelope.[17] In particular, PEDV spike proteins can be divided into two domains with distinct functions: the N-terminal S1 subunit responsible for receptor binding, and the C-terminal membrane anchored S2 domain responsible for membrane fusion.[5,6,10] First reported in England in 1971,[6] it caused mass epidemics in Europe for 1970-1980s. It spread out in Asian countries and exploded in Korea in the 1990s.[9]

Recently, it has become a more important pathogen because the virus occurring endemically in Asian gets more acute and severe.[18-20] It has also severely affected the United States, and led to significant economic losses in the pig industry, even they have adopted an import restriction way.[1,14] Since the explosive PED outbreak in the USA, showing high morbidity and mortality, PED also severely affected Korean commercial swine farms, leading to significant economic losses in the pig industry.[3]

During 2012 to 2013, 3 and 2 positive samples were detected in suckling (3 out of 440 samples) and weaned (2 out of 88 samples) pigs, respectively.[15] There was no positive sample in gilt and sow groups. No other age group in that time was infected. On the other hand, during 2014 to 2015, all age groups of pigs were infected with PEDV, showing the highest detection rate in Suckling piglets.[15]

In this study, the prevalence and its patterns were verified and compared between 2016 and 2017. Among the samples, PEDV strain from NB farm showed the most serious clinical symptoms. Furthermore, we clarified which PEDVs strains are currently circulating in Korea, and where they were closely related to.

MATERIALS AND METHODS

The intestine samples of pigs showing signs of diarrhea (n = 642) collected January 2016 to May 2017 were screened for the presence of porcine epidemic diarrhea virus from 59 commercial farms in 9 provinces. All these samples were randomly collected from commercial swine farms in nine provinces of South Korea. Age groups ranged from suckling to sow pigs. Samples were eluted in PBS, pH 7.2, and stored at -20 ^{o}C until use. This study employed reverse transcriptase - PCR to detect PEDV in intestine samples.

Total RNA was extracted by using Trizol LS (Invitrogen, USA) following the manufacturer's instructions. The RNA was then converted into cDNA with the use of random hexamers and commercial RNA to cDNA EcoDry Premix kit (Clontech, Japan) following the manufacturer's protocol. Finally, PCR reactions were performed with pathogen-specific primers using AccuPower® Pro-Fi Taq PCR PreMix (Bioneer Ltd., Korea). To enhance the sensitivity and specificity for confirmed, we used one pairs PEDV primer

Table 1. *Numbers of PEDV positive samples and detection rates in respective age groups according to researched periods, 2016~2017*

2016-2017 year	Suckling	Weaned	Grower	Finisher	Gilt	Sow	Total
Number of samples	199	84	35	41	154	129	642
Positive samples	18	3	2	15	1	4	43
%	9.05%	3.57%	5.71%	36.59%	0.65%	3.1%	6.70%

†Samples were sorted into 6 groups: female (gilt and sow), suckling (<30 d), weaned (30–60 d), grower (60–90 d); and finisher (≥90 d).

sets. The PEDV-specific primers were designed as PEDV-460F [5′- AATGGCAACAACAGGTCC -3′], and PEDV-947R [5′-GCATCAACACCTTTTTCGAC-3′], which amplified 488 bp region of the nucleocapsid protein coding gene.

The thermal profile was initial denaturation at 95oC for 5 min, followed by 35 cycles of 95 oC for 30 s, 56 oC for 30 s, 72 oC for 30 s, and a final extension at 72 oC for 7min.

Total detection rates of the 2016 ~ 2017 period were 6.70% (43 positive samples / 642 total samples). All age groups were infected with PEDV, showing the highest detection rate in finisher group of 36.59% (15/41). In detail, according to age groups: suckling 9.05% (18/199), grower 5.71% (2/35), weaned 3.57% (3/84), sow (3.1%), and gilt 0.65% (1/154) were detected respectively (Table 1). The epidemiological difference of PEDV detection between the age groups were clearly presented.

During 2016 to 2017, all age group's positive rates showed patterns similar to 2015,15 but the finisher group showed the highest positive rate 36.59 %, unlike 2015, which showed highest positive rate 27.17% in suckling group (Table 1). Even pigs over the weaning period showed watery diarrhea symptoms, which is one of the typical signs of PEDV infection in young pigs. Moreover, considering the pattern of the positive rates in the same farms, once PED takes place in a barn, it usually affects to all age groups of pigs in the farm. These facts emphasize the importance of the biosecurity and hygiene management.

We focus on showing severe clinical symptoms of NB farm intestine sample among collected positive samples. On February 21, 2017, a 20-day-old pig in NB farm (Gyungbuk province, South Korea) was sent to the Department of Veterinary Medicine Virology Laboratory at Seoul National University for the investigation of possible cause(s) of death. NB farm is under single-site production system (farrow-to-grower unit and a grower-to-finish unit).

It was described that the suckling group of the same ban exhibited the same symptoms of diarrhea.

The mortality rates were estimated approximately 100%. The intestine sample (n=1) collected on the NB farm showed PDCoV, rotavirus, kobuvirus, and Transmissible Gastroenteritis Coronavirus were all negative.

The PEDV positive NB farm intestine sample had tested enteric viruses of porcine deltacoronavirus (PDCoV), Transmissible Gastroenteritis Coronavirus (TGEV), Kobuvirus, and Rotavirus. For detecting PDCoV, PDCoV-587F [5′- CCCAGCTCAAGGTTTCAGAG -3′] and PDCoV-587R [5′- CCCAATCCTGTTTGTCTGCT-3′], were utilized, which amplified 587 bp region of the nucleocapsid protein coding gene,15 TGEV were detected by using i-TGEV/PEDV Detection kit (iNtRON., Korea), and Kobuvirus using generic kobuvirus primers (UNIV-kobu-F/UNIV-kobu-R).16 Rotavirus using detected primer sets (rot3 and rot 5) followed the protocol's.[12]

Immunohistochemistry (IHC) was performed in the pig intestine to conform PEDV infection of NB1 strain; the following intestine tissue was collected at 10% neutral buffered formalin. After 1 day of fixation, samples underwent dehydration through graded alcohols and a toluene step, and were embedded in paraffin wax. Sections were then cut at a thickness of 5μm and mounted on "Superfrost/plus" slides (Fisher Scientific, Pittsburgh, PA, USA).

Deparaffinization occurred with three changes of xylene for 5 min each followed by rehydration with 100% alcohol, 95% alcohol, 90% alcohol, 80% alcohol, 70% alcohol, tap water, and distilled water. Approximately 300 μl of monoclonal primary antibody specific for the nucleo protein of PEDV were designed with an "anti-PEDV N antibody kit from MYBioSource Ltd., (Cat No. MBS560870), diluted 1:100, applied to each slide, then transferred to a 37℃ incubator for 1 h, removed and secondary antibody "anti-Mouse IgG-HRP kit from GenDEPOT Ltd., (Cat No.Sa001-500) incubator for 1 h. Colorization was utilized to the ImmPACTTM DAB kit (VECTOR LABORATORIES., Burlingame, CA, USA), according to the manufacturer's instructions. The slides were then counterstained with hematoxylin, dehydrated, and cover-slipped. Immunohistochemistry (IHC) performed in pig intestine for NB1 strain is presented in Supplementary Figure 1. In immunohistochemistry (IHC) result, the virus was usually infected in villous enterocytes showing high a positive brown signal by IHC. Exfoliation and vacuolation of enterocytes were seen on the tips of villi or spread over the entire villi. Negative control not showed specially brown color. For the genetic characterization of PEDV causing severe diarrhea in NB farm, we performed a complete sequencing.

The full-length genome was sequenced by a primer walking method, which utilized 26 overlapping primer pairs.8 In particular, the amplifying of the S and N gene primers cannot be performed.[8] (Author, lease check the re-wording of this sen-

Table 2. *Nucleotide and amino acid sequence identities (%) of different regions of the NB1 genome compared with those of the other viruses between Korea, China, and the United States*

		Identity to NB1 / Nucleotide (Amino Acid)							
Reference strains		Complete genome	ORF1a	ORF1b	S	ORF3	E	M	N
Attenuated DR13		96.8 (-)	97.2 (98.2)	97.3 (99.1)	93.2 (94.3)	90.4 (91.6)	87.0 (88.3)	97.8 (99.6)	95.9 (97.7)
SM98		96.3 (-)	96.4 (97.5)	97.3 (98.9)	92.2 (93.3)	92.4 (92.9)	95.2 (96.1)	96.2 (97.4)	94.9 (96.8)
KNU1305		99.8 (-)	99.4 (99.3)	99.5 (99.6)	99.1 (98.9)	99.0 (100)	99.1 (98.7)	99.6 (99.6)	99.0 (98.9)
CV777		96.8 (-)	97.1 (98.0)	97.2 (99.1)	93.1 (94.2)	90.1 (91.6)	94.8 (97.4)	97.7 (99.1)	95.7 (97.7)
AH2012		99.5 (-)	99.1 (99.1)	99.4 (99.6)	98.5 (98.9)	98.4 (100)	98.3 (98.7)	99.3 (99.6)	98.5 (98.6)
JS2008		97.0 (-)	97.3 (98.3)	97.6 (99.1)	93.4 (94.7)	90.1 (91.6)	95.2 (96.1)	97.7 (99.1)	95.9 (97.7)
Indiana34		99.8 (-)	99.4 (99.2)	99.5 (99.6)	98.7 (98.6)	99.4 (100)	99.1 (98.7)	99.6 (99.6)	99.0 (98.9)
Colorado30		99.7 (-)	99.2 (99.1)	99.5 (99.6)	99.0 (99.0)	99.4 (100)	99.1 (98.7)	99.4 (99.6)	99.0 (98.8)
Texas31		99.7 (-)	99.2 (99.2)	99.5 (99.6)	99.1 (99.1)	99.4 (100)	99.1 (98.7)	99.6 (99.6)	98.9 (98.6)
PC21A		99.2 (-)	99.3 (99.2)	99.3 (99.6)	99.2 (99.1)	99.4 (100)	99.1 (98.7)	99.6 (99.6)	99.0 (98.9)

Figure 1. *Bayesian time- scaled phylogeny (BSP) of PEDV with inferred geographical location states. The round branches of the maximum clad credibility tree were colored according to the most probable location state of their descendent nodes. The locations of Korean strains were indicated gray color, and NB1_2017_korea appeared red square box.*

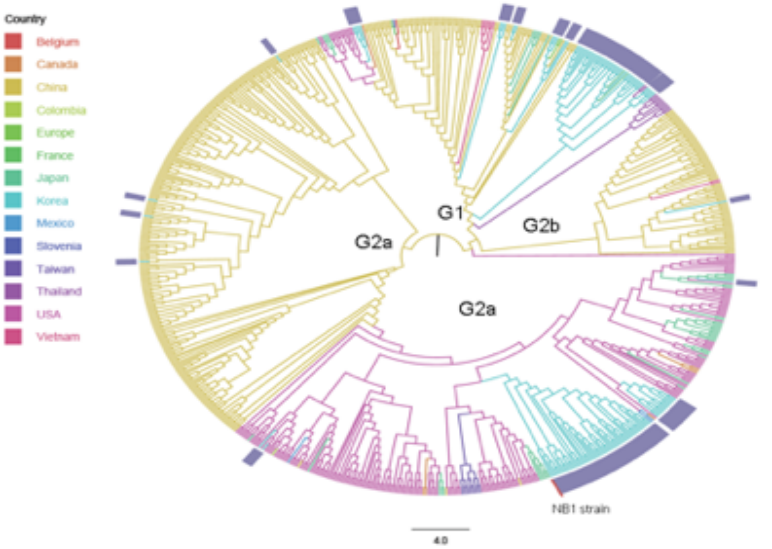
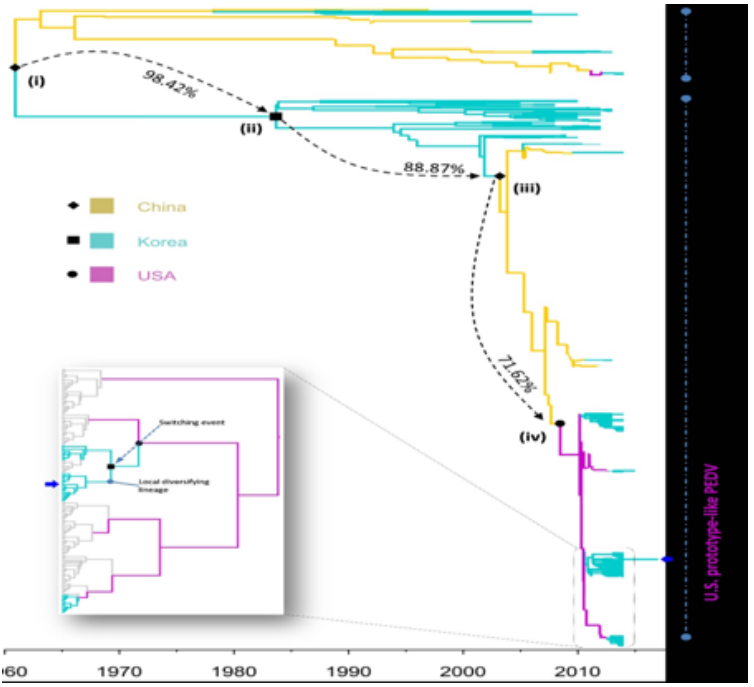


Figure 2. *Bayesian time- scaled phylogeography of PEDV*
The branches of the maximum clade credibility tree were colored according to the most probable location state of their descendent nodes. For clarity, branches did not relate to Korean PEDVs were made invisible. Dash arrows indicated the transitions of root location states [labeled from (i) to (iv)] which were supported by high posterior probabilities. Inserted panel was a cladogram of PEDVs leading to NB1 strain (indicated by a filled arrow).



tencs) In our previously published paper,[3] we outlined the conditions of the Technical Appendix (Table 1). The specific PCR products were purified by using the gel extraction method and further processed for TA cloning and transformation.[17] The full-length genome of strain NB1_2017_Korea (Accession number. MF281416) size of 28,005bp. The genome arrangement and corresponding nucleotide positions are as follows:

- 5' untranslated region (UTR), nt 1to 266
- replicase, nt 266 to 12,574 for 1a, and nt 12,574 to 20,610 for 1b
- spike (S), nt 20,607 to 24,764
- open reading frame 3 (ORF3), nt 24,764 to 25,438
- envelope (E), nt 25,419 to 25,649
- membrane (M), nt 25,657 to 26,337
- nucleocapsid (N), nt 26,349 to 27,674, and
- 3'untranslated region, nt 27676 to 28,005.

The complete PEDV NB1_2017_Korea strain belongs to genogroup 2a (KNU 1,305, AH 2012, Indiana 34, Colorado 30, Texas 31, PC 21A), which has a nucleotide identity of 99.2 to 99.9% between strains and 96.3 to 97% for genogroup 1 strains (attenuated DR 13, SM 98, CV 777, JS 2,008). The ORF1a b, S, ORF3, envelop (E), membrane (M) gene, and N genes showed different patterns between genogroups 1 and 2. However, the membrane (M) gene of attenuated DR13 (G1a) showed highest amino acid identity of 99.6% (Table 2).

Interestingly, there was a significant genetic difference of S gene from the currently circulating virus strains (G2), NB1_2017_Korea (G2a) strain had deleted in 429-431 part nucleotide sequences ATA (N amino acid) which is similar to genogroup 1 strains (Supplementary Figure 2). In the Supplementary table, amino acid substitution of mainly structure protein coding genes (ORF3, S, N, E, and M), the ORF3 1 (E30D), S 28 (X117Q, L183S, K196N, S253R, H327Q, S365E, I388F, F389L, Y395N, L415P, C427F, L487H, X491Y, H547Y, X587Y, V610F, X637C, Y664N, H664L, I679L, T683I, V684F, L687F, E782K, R842H, P997S, Q1060S, W1090R), N 9 (D27E, D47E, P162Q, D220Y, A224S, L242F, Q250R, E251V, P261V), E 2 (M64L, I74T), and M 1 (A42V) coding genes substitution have showed comparable to the present circulating strain of USA_Colorado strain (G2a) (KJ645638).

RESULTS

In order to infer where the most probable geographical region of NB1 was, Bayesian geographical analysis13 was applied to the coding gene of the complete S gene and S1 domain.

This coding region suggested that it was appropriate to study the genetic relatedness of PEDVs2 and the S1-based phylogeny nearly resembled the full S gene-based phylogenetic tree.[11] The data set for this analysis was 812 sequences deposited in GenBank, which contians collection date and country of origin. The settings of Bayesian phylogeographic analysis using BEAST package[7] were followed the previous publication.[4]

As depicted in Figure 1, the PEDV genotypes clearly distributed 1 and 2. Since 2013, isolates of Korea, China, the United States, and Japan could be cleary divided 2a and 2b genotypes. Geno group 1 strains emerged only from 1996 to 2012 in the world. As shown in Fig 1, NB1 strain was inferred to originate from Korea, while the recent Korean PEDVs were derived from China and USA. The Korea clade, included in Geno group 2a, showed its own derivation. For the S complete gene of PEDV, geometric mean evolutionary rates at 95% highest posterior density (HDP) ranging from 3.28 x 10-4 to 7.66 x 10-4 nucleotide substitutions per site per year was estimated.

The S1- based phylogeography (Figure 2) showed that PEDV could be divided into genogroup 1 (G1) and genogroup 2 (G2), of which Korean PEDVs were within both G1 and G2. The Bayesian phylogeographical

analysis also captured three main- consecutive switches in root location states [from (i) to (iv), (Figure (Author, please confirm what Figure Number belongs here)]: from China to Korea, Korea to China and China to USA. Focused on the NB1 strain, the virus belonged to G2 and was grouped within US prototype- like PEDVs. However, it directly derived from a local lineage, which was a result of viral diversification upon introduced into Korea (inserted panel, Figure 2). Since 2013, new emerging pattern of PEDV was detected in Korea, of which there were a mixture of classical genogroups (G1 and G2b), and pandemic G2a. That newly introduced pandemic PEDVs were predicted to derive from China and USA.[4] By application of Bayesian phylogeographical analysis, this study also successfully reconstructed diffusion pathways of PEDV into Korea (Figure 2). Interestingly, though grouped within US prototype- like PEDVs, NB1 strain directly originated from a local lineage (inserted panel, Figure 2). The result implied that upon introduced into Korea, PEDV diversified locally and might form a unique lineage.

SUMMARY

In summary, this study demonstrated, after the PED outbreak in the United States, PED also explosively occurred in Korea. The current strains in Korea would be highly similar to those in the United States, throwing the questions about its epidemiological sources. Although strains between Korea and United the States were genetically closely related, prevalence and virulence patterns were clearly different, which meant that the other factors such as the environment or vaccination could be associated. Therefore, additional epidemiological studies should be performed.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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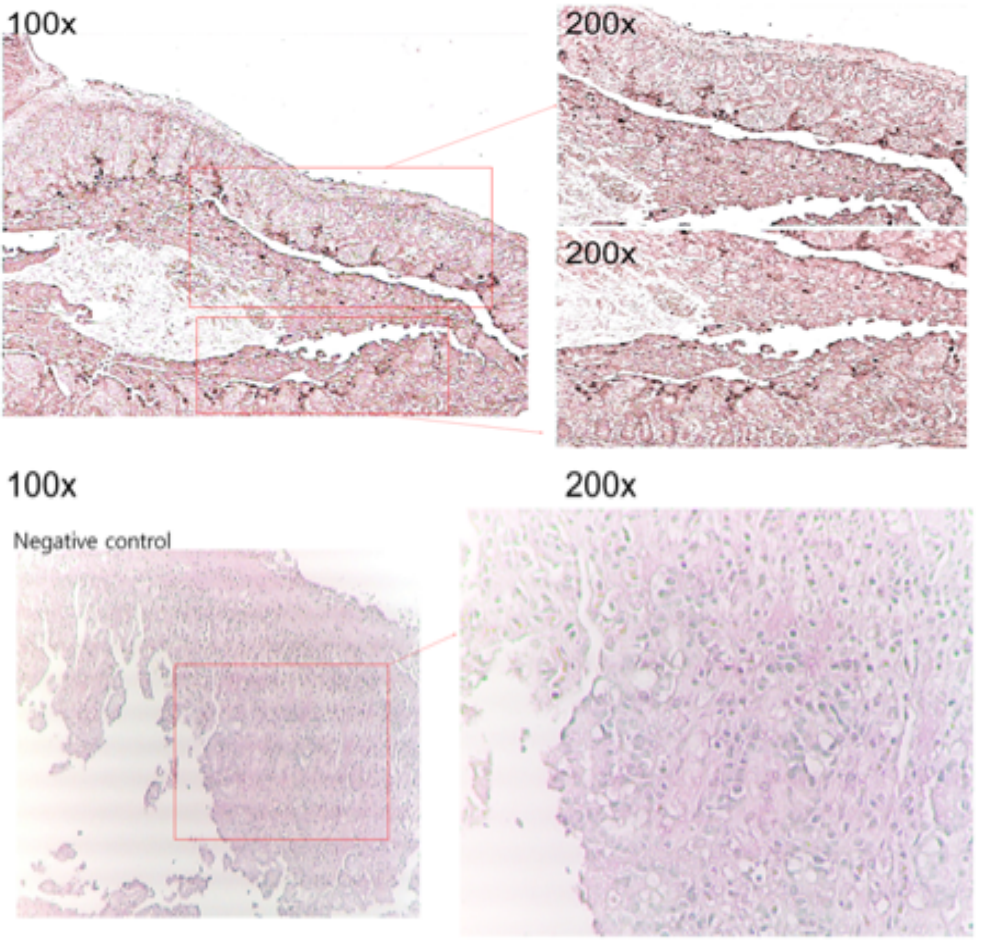
Supplementary Table. *Detailed information about variant regions of amino acid substitutions made in mainly protein genes (ORF3, S, N, E, M) between USA colorado and NB1 strains.*

Genes	USA_Colorado (KJ645638)	Amino acid region	NB1 (MF281416)
ORF3	E	20	D
S	X	117	Q
	L	183	S
	K	196	N
	S	253	R
	H	327	Q
	S	365	E
	I	388	F
	F	389	L
	Y	395	N
	L	415	P
	C	427	F
	L	487	H
	X	491	Y
	H	547	Y
	X	587	Y
	V	610	F
	X	637	C
	Y	664	N
	H	665	L
	I	679	L
	T	683	I
	V	684	F
	L	687	F
	E	782	K
	R	842	H
	P	997	S
	Q	1060	S
	W	1090	R
N	D	27	E
	D	47	E
	P	162	Q
	D	220	Y
	A	224	S
	L	242	F
	Q	250	R
	E	251	V
	P	261	V
E	M	64	L
	I	74	T
M	A	42	V

Supplementary Figure 1. *Immunohistochemistry assay targeting the nucleocapsid protein coding gene of PEDV in the porcine intestine from the NB domestic farm; red box area enlarged x 200 times. Negative control; tissue sections from a healthy pig (8 weeks old), which was RT-PCR negative for PEDV.*



Supplementary Figure 2. *Comparison between NB1_2017_Korea strain and nine PEDV strains of the S1 gene (Gen Bank accession No. JQ023162, GU937797, KJ662670, KC210145, KC109141, KJ645641, KJ645638, KJ645639, and KR078299).*