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BRIEF REPORT

A novel strain of porcine deltacoronavirus in Vietnam

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Abstract Two porcine deltacoronavirus (PDCoV) strains (Binh21 and HaNoi6) were isolated from two pig farms in North Vietnam. Phylogenetic analysis of the complete genomes and the *Spike* and *Membrane* genes revealed that the two Vietnam PDCoVs belong to the same lineage as PDCoVs from Thailand and Laos; however, the *N* genes belonged to the same lineage as PDCoVs from the USA, Korea, China, and Hong Kong. The recombination detection program subsequently identified the major parent (S5011 strain) and minor parent (HKU15-44 strain) of the two Vietnam PDCoV strains ($p < 0.01$).

Family *Coronaviridae* is divided into four genera: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus* [20]. Porcine deltacoronavirus (PDCoV) is an enveloped, single-stranded, positive-sense RNA virus with a genome approximately 25 kb in length. PDCoV comprises the 5′-untranslated region (UTR), open reading frames (ORFs) ORF1a and ORF1b, genes corresponding to the following proteins: spike (S), envelope (E), membrane (M), non-structural protein 6 (Nsp6), nucleoprotein (N), non-structure protein 7 (Nsp7); and finally the 3′-UTR [21]. The S glycoprotein contains two domains, S1 and S2, which play an important role in binding to specific host cell receptors [20]. Clinical observations in experimentally infected gnotobiotic and conventional nursing pigs have revealed that PDCoV induces acute, watery diarrhea, which is often accompanied by acute mild to moderate vomiting, ultimately leading to dehydration, loss of body weight, lethargy, and death [1, 4, 11]. PDCoV was first identified in pig samples collected in Hong Kong in 2012 [21]. Emergence of PDCoV on pig farms in the USA was reported in 2014 and rapidly spread across the country [7]. Since then, the virus has been detected in Korea, China, Thailand, and Lao PDR [2, 6, 9, 15]. Phylogenetic analysis of the *S*, *M*, and *N* genes from two strains (P29_15_VN_1_1215 and P30_15_VN_1215) detected on pig farms in Dong Nai and Baria provinces in South Vietnam revealed that they belonged to the Hong Kong, Korea, and USA lineages [14].

The objective of the present study was to investigate circulating PDCoV strains in North Vietnam and to analyze their complete genome sequences. In October and December 2015, two cases of diarrhea were reported on pig farms located in Phu Xuyen-Ha Noi province and Thai-Thuy-Thai Binh province, both of which are located in northern Vietnam. The pig farm in Phu Xuyen-Ha Noi bred a farrow-to-finisher herd of 634 head. Clinical symptoms included vomiting and watery diarrhea, resulting in the death of some piglets less than 30 days old. Morbidity was 100% (80/80) for piglets, 93.8% (60/64) for weaning pigs, 72.3% (340/470) for growing pigs, and 65% (13/20) for gilts and sows. Mortality was 36.2% (29/80) for piglets and 13.3% (8/64) for weaning pigs; none of the growing pigs, gilts, or sows died. The pig farm in Thai-Thuy-Thai Binh province

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bred about 5,000 head, all aged from 2 to 6 months. Clinical symptoms were noted for about 14 days, and pigs of all ages were infected and had diarrhea. Morbidity was about 65% (3,250/5,000); however, the only clinical sign was diarrhea and no pigs died. Diarrheal fecal samples (n = 26) collected from the two pig farms were subjected to RT-PCR to test for the *Spike* (*S*) gene of porcine epidemic diarrhea virus (PEDV), the *Nucleocapsid* (*N*) gene of transmissible gastroenteritis virus (TGEV), and the *N* gene of PDCoV, as previously described [5, 13, 19]. Viral RNA was extracted from feces using TRIzol LS[b], according to the manufacturer's instructions, and products of the expected size were cloned into the pGEM-T Vector System II (Promega, Cat. No. A3610, USA). The cloned gene was then sequenced on an ABI Prism® 3730xl DNA Sequencer at the Macrogen Institute (Macrogen Co., Ltd.) using T7 and SP6 sequencing primers. Ten fecal samples from 21-day-old piglets at the farm in Phu Xuyen-Ha Noi were collected in October 2015, and all were positive for PDCoV; while none were positive for PEDV or TGEV. Sequence analysis of the PDCoV *N* genes from the ten samples revealed 99.8–100% identity. Sixteen fecal samples (from pigs aged 2 to 5 months) were collected from Thai Thuy-Thai Binh in December 2015, and 14 were identified as PDCoV-positive by RT-PCR; while none were positive for PEDV or TGEV. The PDCoV *N* genes from these 14 samples were 99.7–100% identical at the nucleotide (nt) level. We sequenced the complete genome of one (HaNoi6) of the ten strains detected at Phu Xuyen-Ha Noi and one (Binh21) of the 14 strains detected at Thai-Thuy-Thai Binh and submitted them to GenBank under accession numbers KX834351–KX834352. The complete genome sequences were then aligned using the CLUSTALX alignment program [17].

The HaNoi6 and Binh21 strains are 25,406 nt in length and 99.9% identical at the nt level. The ORF1a/1b and *S* genes of HaNoi6 and Binh21 are 99.9% and 99.8% identical, respectively, whereas the 5' and 3' UTR and *E, M, N, NSP6, NSP7* genes are 100% identical (Table 1). The complete genome of Binh 21 strain showed 98.4% identity at the nt level with the S5011 and TT_1115 stains from Thailand and the P1_16BTL_0115 strain from Laos (Table 1). The *S* genes of the Binh21 and HaNoi6 strains were 99.8% identical at the nt level, but only 96.6–97.1% identical to strains from the USA, Korea, China, and Thailand. Interestingly, the S protein of Binh21 was 98.1–98.3% identical at the amino acid level to strains HKU15-44, Minnesota 159, and KNU14-04, but only 97.4% identical to strains from Thailand and Laos (Table 1). However, phylogenetic analysis of the S gene/protein at the nucleotide and amino acid level revealed that Vietnam Binh21 and HaNoi6 strains were included in the same group with Thailand and Laos strains. The reason for this discrepancy between amino acid identity and phylogenetic tree analysis for the S gene is not clear, but it might be due to several unique sequences that determine each cluster on the phylogenetic tree. The amino acid sequences of the E and M proteins of HaNoi6 and Binh21 are 100% identical to those of the S5011, CH/S27, HKU15-44, Minnesota159, and KNU14-04 strains. The E and M proteins are transmembrane proteins associated with viral envelope formation and release [20]. Compared with the HKU15-44 strain, the HaNoi6 and Binh21 strains lack a three nt (CTA) segment in the 5' UTR (110–112 bp), six nt (TTTGAA) and nine nt (GCCGGTTGG) segments in ORF1a/1b (1,732–1,737 bp and 2,803–2,811 bp, respectively), and have a single nt (C) inserted in the 3' UTR (25,042 bp). Also, compared with the S5011 strain, the HaNoi 6 and Binh21 strains have a single

Table 1 Comparison of the nucleotide sequence of Binh21 strain with those of reference viruses obtained from GenBank

Strains	Nucleotide sequence/amino acids sequence homology (%)									
	Full genome	5' UTR	ORF1a/1b	S	E	M	N	NSP6	NSP7	3' UTR
HaNoi6	99.9	100	99.9 /99.9	99.8 /99.8	100 /100	100 /100	100 /100	100 /100	100 /100	100
TT_1115	98.4	98.8	98.8 /99.0	96.8 /97.4	99.6 /100	98.7 /99.5	97.9 /98.5	98.9 /98.9	97.0 /92.0	96.9
S5011	98.4	98.6	98.8 /98.9	96.9 /97.4	99.6 /100	99.0 /100	97.0 /98.8	98.9 /98.9	97.1 /92.5	96.9
BTL_0115	98.4	98.6	98.7 /97.8	96.9 /97.4	99.6 /100	99.0 /99.5	98.1 /98.5	98.9 /98.9	97.3 93.0	96.9
CH/S27	98.0	98.8	98.1 /98.7	96.6 /97.9	98.8 /100	99.2 /100	98.9 /99.4	98.9 /97.8	98.6 /96.0	98.4
HKU15-44	97.9	98.8	97.5 /97.8	97.1 /98.1	99.6 /100	99.5 /100	99.2 /99.7	99.2 /98.9	99.1 /97.5	98.7
Minnesota159	97.7	98.6	97.4 /98.1	96.8 /98.3	99.2 /100	99.2 /100	98.9 /99.7	98.9 /97.8	98.6 /96.0	98.4
KNU14-04	97.7	98.7	97.3 /98.0	96.8 /98.3	99.2 /100	98.9 /100	98.9 /99.7	98.9 /97.8	98.8 /96.5	98.2

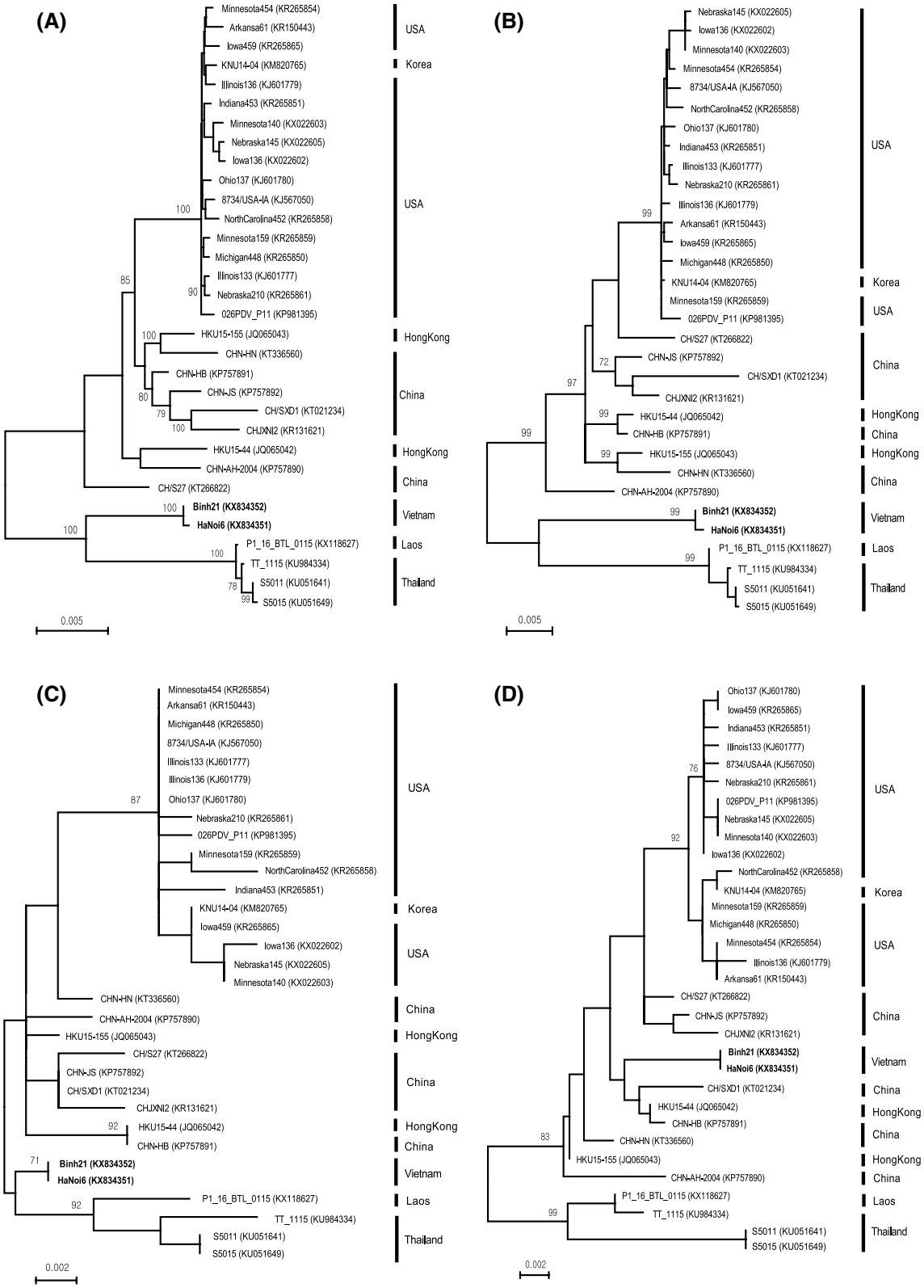

Fig. 1 Phylogenetic analysis of 32 PDCoV strains based on the nucleotide sequences of the complete genome (A) and the *S* (B), *M* (C), and *N* (D) genes. The trees were constructed using the ML method (based on the Tamura-Nei model) and bootstrap analysis (n= 1,000) in Mega 6.06 software. Bootstrap values >70% are shown at the branch points. The scale bar indicates the number of nucleotide substitutions per site. The two Vietnamese PDCoV strains are marked in bold

Fig. 2 Recombination analysis of the Binh21 (A) and Hanoi6 (B) strains. Possible breakpoints in the recombination event between S5011 and HKU15-44 were estimated using the RDP method and confirmed by the BootScan, MaxChi, and Chimaera applications in the RDP program ($p < 0.01$)

nt (A) inserted in the 5' UTR (298 bp). After Clustal X multiple sequence alignment, three phylogenetic trees representing complete genome sequences as well as sequences of the S and M genes of 32 PDCoV strains from GenBank, including the two Vietnam PDCoV strains identified herein, were generated using the maximum-likelihood (ML) method, with the Tamura-Nei model and bootstrap analysis (n = 1000). This was performed using the MEGA 6.06 software with default parameters [16]. Phylogenetic analysis of the complete genome, S gene, and M gene sequences showed that the two Vietnam PDCoV strains belong to the same lineage as three Thailand PDCoVs and one Laos PDCoV (Fig. 1A–C). Analysis of Thailand's PDCoV strains suggested that they clustered as a novel lineage of PDCoV that was separate from the US-like and China-like lineage PDCoVs [8]. The finding also suggested that the Thailand PDCoV isolates could have evolved from the same ancestor as other PDCoV strains but had differentiated at some point in the past [8]. The origin and source of the PDCoVs introduced into North Vietnam is currently unknown, but phylogenetic analysis suggests that Hanoi6 and Binh21 are closely related to Thailand and Laos strains, a finding supported by the close geographic proximity of these regions. However, the ML tree revealed that the N genes of Hanoi6 and Binh21 belong to the same lineage as strains from the USA, Korea, China, and Hong Kong (Fig. 1D). The N protein is involved in viral replication, function, and pathogenesis [12]. Therefore the two Vietnam PDCoV strains were analyzed using the recombination detection program (RDP). The recombination breakpoint was determined using the RDP method and confirmed statistically using the BootScan, MaxChi, and Chimaera applications ($p < 0.01$). The major parent and minor parent were identified as S5011 and HKU15-44, respectively ($p < 0.01$). BootScan analysis predicted the potential breakpoints in HaNoi6 and Binh21 to be at nt 1,007 and nt 23,846 (Fig. 2). Recombination events are often reported in PEDV studies, and the majority are intra-recombinants of different lineages of the same type of enteric coronavirus [3, 18]. A recent study of possible recombination events in PDCoV strains reported that the CHN-GD16-05 strain belonged to American and Korean lineages, while the CHN-GD16-03 strain was similar to a

Thailand strain, but only in terms of the *S* gene [10]. The recently emerging PDCoV strains in Thailand are highly virulent, causing a mortality rate of 19.22% in pig farms [2]. The HaNoi6 strain, belonging to the same lineage as Thailand PDCoV strains, has a mortality rate in piglets and weaning pigs of 36.2% and 13.3%, respectively. HaNoi6 was also observed in gilts and sows, indicating that the pathogenicity of PDCoV is not confined to piglets.

In conclusion, genetic analyses, based on complete genome sequences, demonstrated that HaNoi6 and Binh21 strains from North Vietnam are similar to PDCoV strains from Thailand and Laos. Like Thailand's PDCoV strains, the HaNoi6 strain is highly virulent and pathogenic to piglets. Recombination events in the two Vietnamese PDCoV strains were identified in the *N* gene region. The genetic lineages of PDCoV strains from different countries appears to differ, suggesting that they may recombine and evolve continuously to generate diverse genotypes.

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