RAPID COMMUNICATION

Detection and Phylogenetic Analysis of Porcine Deltacoronavirus in Korean Swine Farms, 2015

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Keywords: porcine deltacoronavirus; swine; South Korea

Summary
This study applied molecular-based method to investigate the presence of porcine deltacoronavirus (PDCoV) in 59 commercial pig farms in South Korea. The results of RT-PCR screening on a relatively large collection of faeces samples (\(n = 681\)) from January 2013 to March 2015 did not reveal the presence of PDCoV until the end of 2014. However, on March 2015, PDCoV-positive samples (SL2, SL5) were detected from SL swine farm in Gyeongbuk province. The phylogenetic trees based on the complete spike- and nucleocapsid protein-coding genes showed that SL2 and SL5 closely related to the US PDCoV strains rather than those in China. Thought Korean strains of PDCoV isolated in 2014 (KNU14.04) and in 2015 (SL2 and SL5) grouped within US PDCoV cluster, the reconstruction of ancestral amino acid changes suggested that they are different.

Introduction
Coronaviruses are single-stranded, positive-sense enveloped RNA viruses belonging to the \textit{Coronaviridae} family and are divided into 4 genera (\textit{Alphacoronavirus}, \textit{Betacoronavirus}, \textit{Gammacoronavirus}, and \textit{Deltacoronavirus}) (Woo et al., 2012). Until 2014, three members of the \textit{Alphacoronavirus} genus such as porcine epidemic diarrhoea virus (PEDV), transmissible gastroenteritis virus (TGEV) and porcine respiratory coronavirus (PRCV) are known to cause enteric and respiratory diseases of swine. More recently, a novel emerging porcine deltacoronavirus (PDCoV) was demonstrated to be enteropathogenic and causes severe diarrhoea resemble those of PEDV and TGEV infections (Chen et al., 2015; Jung et al., 2015), and mild interstitial pneumonia (Ma et al., 2015). Since the first report of PDCoV in Hong Kong in 2012 (Woo et al., 2012), the virus is identified in the United States (Wang et al., 2014a,b), South Korea (Lee and Lee, 2014) and China (Song et al., 2015). In this study, we further report the presence and genetic characterization of PDCoV from cases showing symptoms of diarrhoea in Korean swine farms.

Materials and Methods
Molecular detection
In this study, faecal samples of pigs showing signs of diarrhoea (\(n = 681\)) collected from January 2013 to
March 2015 were screened for the presence of porcine deltacoronavirus (PDCoV). The sampling locations were given in the Fig. S1. Total RNA was extracted 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, Otsu, Japan) following the manufacturer’s protocol. To enhance the specificity, two pairs of PDCoV primer were utilized. The first method designed primer set of reference (Woo et al., 2012). The other PDCoV-specific primers were designed in this study, targeting a region of 587 bp of the nucleocapsid protein-coding gene (PDCoV-587F 5’- CCCAGCTCAAGGTTTCAGAG-3’, PDCoV-587R 5’- CCCAATCAATCCTGTTTGTCTGCT-3’). The thermal profile was initial denaturation at 94°C for 5 min, followed by 38 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 30 s and a final extension at 72°C for 7 min. The screening for other porcine enteric viruses was performed with pathogen-specific primers using AccuPower® ProFi Taq PCR PreMix (Bioneer Ltd., Daejeon, Korea). For porcine epidemic diarrhoea virus (PEDV) and transmissible gastroenteritis virus (TGEV), we used i-TGEV/PEDV Detection kit (iNtRON Ltd., Daejeon, Korea).

**Table 1.** Results of retrospective detection of PDCoV in NINE provinces from 2013 to March 2015

| Sampling sites | 2013 | 2014 | 2015* |
|---------------|------|------|-------|
|               | n (+) | n (+) | n (+) |
| Gyeonggi      | 78    | 0    | 43    |
| Gangwon       | 46    | 0    | 22    |
| Chungnam      | 46    | 0    | 31    |
| Chungbuk      | 49    | 0    | 22    |
| Jeonbuk       | 38    | 0    | 26    |
| Jeonnam       | 32    | 0    | 18    |
| Gyeongbuk     | 38    | 0    | 22    |
| Gyeongnam     | 22    | 0    | 24    |
| Jeju          | 11    | 0    | 13    |
| Total         | 360   | 0    | 221   |

n, number of faecal samples; +, number of positive samples.

*Until March 2015.

Inferring ancestral amino acid changes

Amino acid changes on the evolutionary path of PDCoV (based on S and N genes) were inferred from a codon-based alignment of 31 sequences of complete S gene (3483 bases) and 31 sequences of complete N gene (1029 bases). The details of the data set are summarized in Table S1. The phylogenetic tree was reconstructed by the maximum likelihood model with 1000 bootstrap replicates implemented in IQ-TREE version 1.3.8 (Nguyen et al., 2015). The best-fitting nucleotide substitution model for each alignment was determined automatically by specifying ‘-m TEST’ option.

**Table 2.** Detection of porcine enteric viruses in diarrhoeal intestinal/faecal samples from pigs of SL farm in March 2015

| Name of samples/Specimens | Clinical symptoms | Pig group* | Collection date | PDCoV | PEDV | TGEV | Group A rotavirus | Kobuvirus |
|---------------------------|-------------------|------------|-----------------|-------|------|------|-------------------|-----------|
| SL1/Faeces                | Diarrhoea         | Sow        | 25 March 2015   | −     | +    | −    | −                 | −         |
| SL2/Faeces                | Diarrhoea, wasted | Finisher   | 25 March 2015   | +     | +    | −    | −                 | −         |
| SL3/Faeces                | Diarrhoea, wasted | Finisher   | 25 March 2015   | −     | +    | −    | −                 | −         |
| SL4/Faeces                | Diarrhoea, wasted | Finisher   | 25 March 2015   | −     | +    | −    | −                 | −         |
| SL5/Intestine             | Acute watery diarrhoea | Suckling | 31 March 2015 | +     | +    | −    | −                 | −         |
| SL6/Intestine             | Diarrhoea         | Suckling   | 31 March 2015   | −     | +    | −    | −                 | −         |

*Pigs were classified into six groups of sow, suckling pigs (<30 days), weaner (30-60 days), grower (60-90 days) and finisher (≥90 days).
Fig. 1. 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 PCDoV isolates in 2014 (KNU14.04) and in 2015 (SL2, SL5) were grouped within US PDCoV cluster, but they located at different branches (highlights).

Fig. 2. The maximum likelihood trees based on the S gene (a) and the N gene (b) with reconstructed non-synonymous substitutions were mapped to the nodes of the phylogeny. For clarity, only branches leading to Korean PDCoV isolates were highlighted (black lines). The nodes where non-synonymous substitutions occurred were indicated by # (for the highlighted branches) and by @ (for the others). The nodes without non-synonymous substitutions were marked by ● (for the highlighted branches) and were not marked (for the others). It was observed that the branch which led to 2015 isolates (SL2, SL5) accumulated further mutations in comparing to the branch which led to 2014 isolate (KNU14.04).
Results and Discussions

The screening results by RT-PCR carried out on 681 samples of 59 swine farms (Table 1) showed that until the end of 2014 all of tests were negative for nucleic acid of PDCoV. It was on March 2015, PDCoV-positive samples were detected in a 600-scale sow farm (SL farm) in Gyeongbuk province. This farm was reported to be infected by PEDV in 2014 and had severe diarrhoea with 100% mortality in piglets. In early 2015, it was observed that up to 20% pigs of all ages had diarrhoea and 10% died. The diagnosis of porcine enteric viruses (Table 2) revealed the dual infection of PDCoV and PEDV, while TGEV, group A rotavirus and Kobuvirus were not detected. In the literature, it was reported that PDCoV co-infected with others enteric viruses, such as: group C rotavirus (Marthaler et al., 2014), TGEV (Dong et al., 2015) and PEDV (Song et al., 2015). Combining the detection results of this study with the above-mentioned reports, it could be inferred that PEDV was the most frequent co-infected viruses.

For the genetic characterization, the maximum likelihood phylogenetic trees reconstructed from the S and N genes (Fig. 1a, b) showed a clear separation between Chinese and US strains of PDCoV and is similar to the previous studies (Marthaler et al., 2014; Wang et al., 2016). Of which, Korean strains of PDCoV isolated in 2014 (KNU14.04) and in 2015 (SL2 and SL5) were grouped within US PDCoV cluster; however, they located at different branches (highlights, Fig. 1a, b). Based on the S gene, the inferred ancestral amino acid changes along the nodes of the phylogeny (Fig. 2a) showed that the branches leading to Korean PDCoV isolates in 2014 and in 2015 shared 1 nucleotide and amino acid sequence and genetic organization of porcine kobuvirus, a member of a new species in the genus Kobuvirus, family Picornaviridae. Arch. Virol. 154, 101–108.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Sampling sites for retrospective detection of PDCoV in 9 provinces from 2013 to March 2015.

Table S1. List of sequences used in this study.

Table S2. List of non-synonymous substitutions at the nodes of the PDCoV phylogeny based on the spike protein coding gene (shown in Figure 2A).

Table S3. List of non-synonymous substitutions at the nodes of the PDCoV phylogeny based on the nucleocapsid-protein coding gene (shown in Figure 2B).