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BRIEF REPORT

Detection, sequence analysis, and antibody prevalence of porcine deltacoronavirus in Taiwan

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Abstract

Porcine deltacoronavirus (PDCoV) was initially documented in Hong Kong and later in the United States, South Korea, and Thailand. To investigate if PDCoV is also present in Taiwan, three swine coronaviruses—PDCoV, porcine epidemic diarrhea virus (PEDV), and transmissible gastroenteritis coronavirus (TGEV)—were tested using real-time reverse transcription polymerase chain reaction (rRT-PCR) in 172 rectal swab samples from piglets exhibiting diarrhea between January 2016 and May 2017 on 68 pig farms in Taiwan. The rRT-PCR results were positive for PDCoV (29/172, 16.9%), PEDV (36/172, 20.9%), TGEV (2/172, 1.2%), and coinfections (16/172, 9.3%). After cloning and sequencing, PDCoV nucleocapsid genes were analyzed. Phylogeny results indicated that the nucleotide sequences of all isolates were like those reported in other countries. To further trace PDCoV in the period of 2011 to 2015, an enzyme-linked immunosorbent assay (ELISA) was used to detect antibodies against PDCoV. The results showed that 279 of 1,039 (26.9%) sera were positive for the PDCoV nucleocapsid protein, implying that PDCoV might have existed in Taiwan before 2011.

Porcine deltacoronavirus (PDCoV) is an enveloped, positive-sense single-stranded RNA virus that belongs to the family *Coronaviridae*. PDCoV was first discovered in Hong Kong, China in 2012 [1]. It was subsequently reported in the United States [2–4] and South Korea [5] in 2014, followed by Thailand and mainland China in 2015 [6, 7]. Currently, there are at least three members of the family *Coronaviridae* that can cause diarrhea in pigs: transmissible gastroenteritis virus (TGEV), porcine epidemic diarrhea virus (PEDV), and porcine deltacoronavirus (PDCoV) [8]. Both TGEV and PEDV belong to the genus *Alphacoronavirus*, whereas PDCoV belongs to the new genus *Deltacoronavirus*. PEDV is an important enteric pathogen that causes piglet diarrhea worldwide, and it has caused significant economic losses in the swine industry in Taiwan from 2013 to 2014 [9]. However, PDCoV and PEDV share a similar clinical manifestation, and many studies have shown that coinfection with PEDV and PDCoV is common in piglets [2–4]. Thus, the purpose of this study was to identify the viruses responsible for causing diarrhea in piglets and to specifically investigate the prevalence of PDCoV infection in Taiwan.

In this study, 172 rectal swabs of piglets that suffered from diarrhea from 68 pig farms located in central and southern Taiwan were collected between January 2016 and May 2017. All rectal swabs were transported in phosphate-buffered saline with 5% glycerol. The total nucleic acid content was then extracted from the rectal swabs using a LabPrep™ DNA/RNA Mini Kit (Taigen Biotechnology, Taiwan). The isolated nucleic acid samples were tested for the presence of swine enteric coronaviruses—TGEV, PEDV, and PDCoV—using the IDEXX™ RealPCR® Test Kits. PDCoV-positive samples were further examined by traditional reverse transcription polymerase chain reaction (RT-PCR) with primers PDCoV-N1 (5'-ACCATCGCTCCAAGTCATTCTTG-3') and PDCoV-N2 (5'-GAGTGGAGTTGGGTG GGTTTA AC-3'). The traditional RT-PCR was done using a LabStar™ OneStep RT-PCR Kit (Taigen Biotechnology, Taiwan). The

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reverse-transcription reaction was performed at 50 °C for 30 minutes, and then a standard polymerase chain reaction was performed: 30 cycles of 94 °C (30 seconds), 55 °C (30 seconds), and 72 °C (1 minute). After electrophoresis and gel elution, amplified products were cloned and sequenced. The nucleotide sequence data were analyzed using Chromas Lite, and the deduced amino acid sequences of the open reading frames were compared to other PDCoV sequences using BLAST. The sequences of three complete PDCoV nucleocapsid (PDCoV-N) genes, Taiwan 22, Taiwan 33, and Taiwan 36, were obtained and deposited in GenBank (accession numbers KY586147 to KY586149). Multiple sequence alignment and phylogenetic tree construction were then performed using the MEGA7 program [10].

A PDCoV-N clone (Taiwan 22) was further subcloned into the protein expression vector pET-32a(+) using primers PDCoV-N-F (5'-ACCGGATCCATGGCTGCACCAGTA GTCCC-3') and PDCoV-N-R (5'-CACAAGCTTCTACGC TGCTGATTCCTGCT-3'). The PDCoV-N protein was then expressed by adding isopropyl-β-D-thiogalactoside (IPTG) (final concentration 0.3 mM) to a bacterial culture and was purified using immobilized metal affinity chromatography for a subsequent enzyme-linked immunosorbent assay (ELISA). A total of 1,039 serum samples collected from 34 pig farms in Taiwan during 2011 to 2015 were tested, and 27 specific-pathogen-free (SPF) pig sera were obtained from the Animal Technology Institute Taiwan to be used negative controls. The ELISA was conducted following the modified procedures described in a previous study [11]. In brief, 96-well microtiter plates were coated with purified His-tagged PDCoV-N (100 ng/well) in 50 mM carbonate buffer (pH 9.6) at 4 °C for 12-14 hours and blocked with blocking buffer (3% bovine serum albumin in Tris-buffered saline (TBS): 50 mM Tris-Cl, pH 7.5, 150 mM NaCl) at room temperature for 1 hour. After washing three times with TBST (TBS containing 0.05% Tween-20), 100 µl of the tested sera (1:100 dilution in blocking buffer) were added to the wells and incubated at room temperature for 1 hour. After incubation, the plate was washed three times with 200 µl of TBST and incubated at room temperature for 1 hour with 100 µl of goat α-porcine Ig antibody conjugated with horseradish peroxidase at a concentration of 1:4000 dilution in blocking buffer. After washing, 100 µl of 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution was added, and the plate was incubated at room temperature for 10 minutes. The development reaction was stopped by adding 50 µl of 2 M H_2SO_4, and the absorbance at 450 nm wavelength was measured. All tests included a blank coated with antigen only, a second antibody control, and six SPF sera as negative controls. If the detection value was lower than ($mean_{neg}$ + 2× SD), the serum was considered negative. On the other hand, a serum detection value larger than [($mean_{neg}$ + SD) × 2] was considered positive.

Of these tested samples, 29 were positive for PDCoV (16.9%), 36 were positive for PEDV (20.9%), and only two were positive for TGEV (1.2%). Regarding coinfection rates, only one of the 172 specimens (0.6%) was positive for all three coronaviruses, one was positive for PEDV and TGEV (0.6%), and 14 of them (8.1%) were positive for PDCoV and PEDV. Based on the real-time RT-PCR (rRT-PCR) detection results, the percentage of pig farms that were positive for at least of one of the coronaviruses was 25% for PDCoV (17/68), 22.1% for PEDV (15/68), and 2.9% for TGEV (2/68).

We compared the sensitivity of the IDEXX rRT-PCR kit and the traditional RT-PCR used for cloning the N protein of PDCoV in this study. All 29 PDCoV-positive samples were re-examined by conventional RT-PCR, which showed that only eight (8/29, 27.6%) were positive, accounting for 4.7% (8/172) of total tested samples. The relatively low positive rate determined by conventional RT-PCR was comparable to that determined by a similar method in a recent study, in which PDCoV was detected exclusively in nursing piglets, with an overall prevalence of approximate 1.28% (5/390) in southern China [12]. It is plausible that for the specimens containing PDCoV genomic RNA of low quantity or quality, most of the commercial rRT-PCR kits, which are designed to amplify short target sequences, can achieve higher sensitivity in detection compared with conventional RT-PCR. In addition, four of the eight positive samples were cloned and sequenced, and the sequence data matched the PDCoV-N reference sequences. All of these four sequences covered the complete coding sequences of PDCoV-N, but one of them, Taiwan 34, had a nonsense mutation within the PDCoV-N gene (data not shown). Such a mutation might arise from an occasional change in RNA sequence occurring during serial propagation of PDCoV [13]. Despite the mutation, the result revealed the presence of the PDCoV genome in the specimen in which Taiwan 34 was cloned, while it remained to be clarified if mutations occurred in other PDCoV-encoded genes as well.

Phylogeny analysis of PDCoV-N genes showed that PDCoVs found in Taiwan were highly similar in their nucleotide sequences to isolates from the United States, mainland China, and other countries (Fig. 1). However, the amino-acid-based phylogeny results of the PDCoV-N proteins revealed that Taiwan isolates can be clustered into different groups (Fig. 2). This might be due to missense mutations in the 66^{th} (F → Y), 198^{th} (E → K), 234^{th} (G → R/T), 271^{th} (F → S), and 275^{th} (G → E/D) amino acid residues of the PDCoV-N protein (Fig. 3). The charges of amino acids were changed in three residues (198^{th}, 234^{th}, and 275^{th}), and the remaining residue changes had altered polarity. The phylogeny results imply that PDCoVs isolated in Taiwan might have existed for a long time.

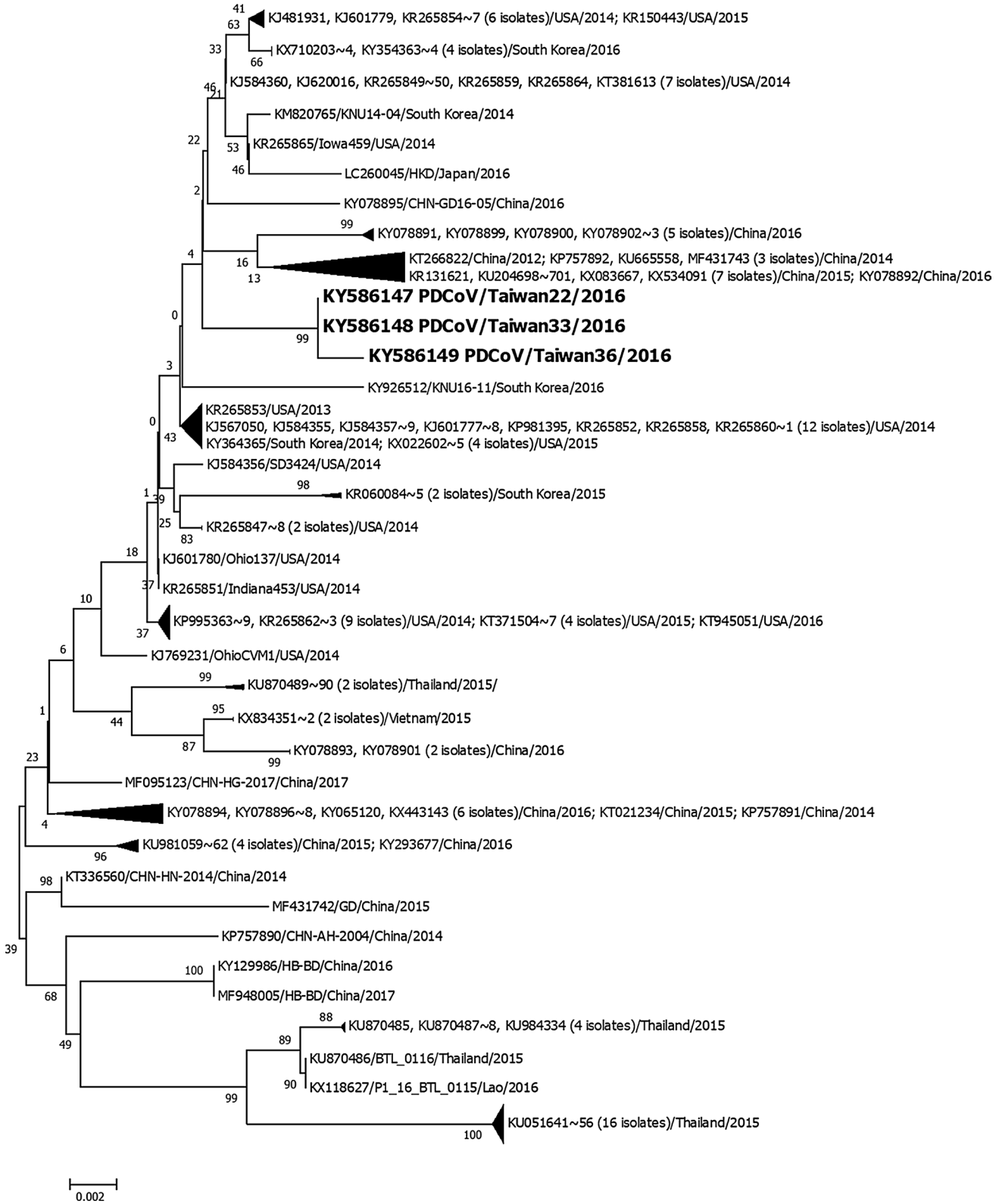

Fig. 1 Phylogenetic tree of the PDCoV-N nucleotide sequences constructed using the distance-based neighbor-joining method in MEGA7 [10]. All analyzed sequences obtained from GenBank were made available in August 2017 and are indicated by their accession number and/or strain name. The sequences obtained in this study are indicated by bold font. Bootstrap values were calculated with 1,000 replicates. The scale bar indicates the number of nucleotide substitutions per site

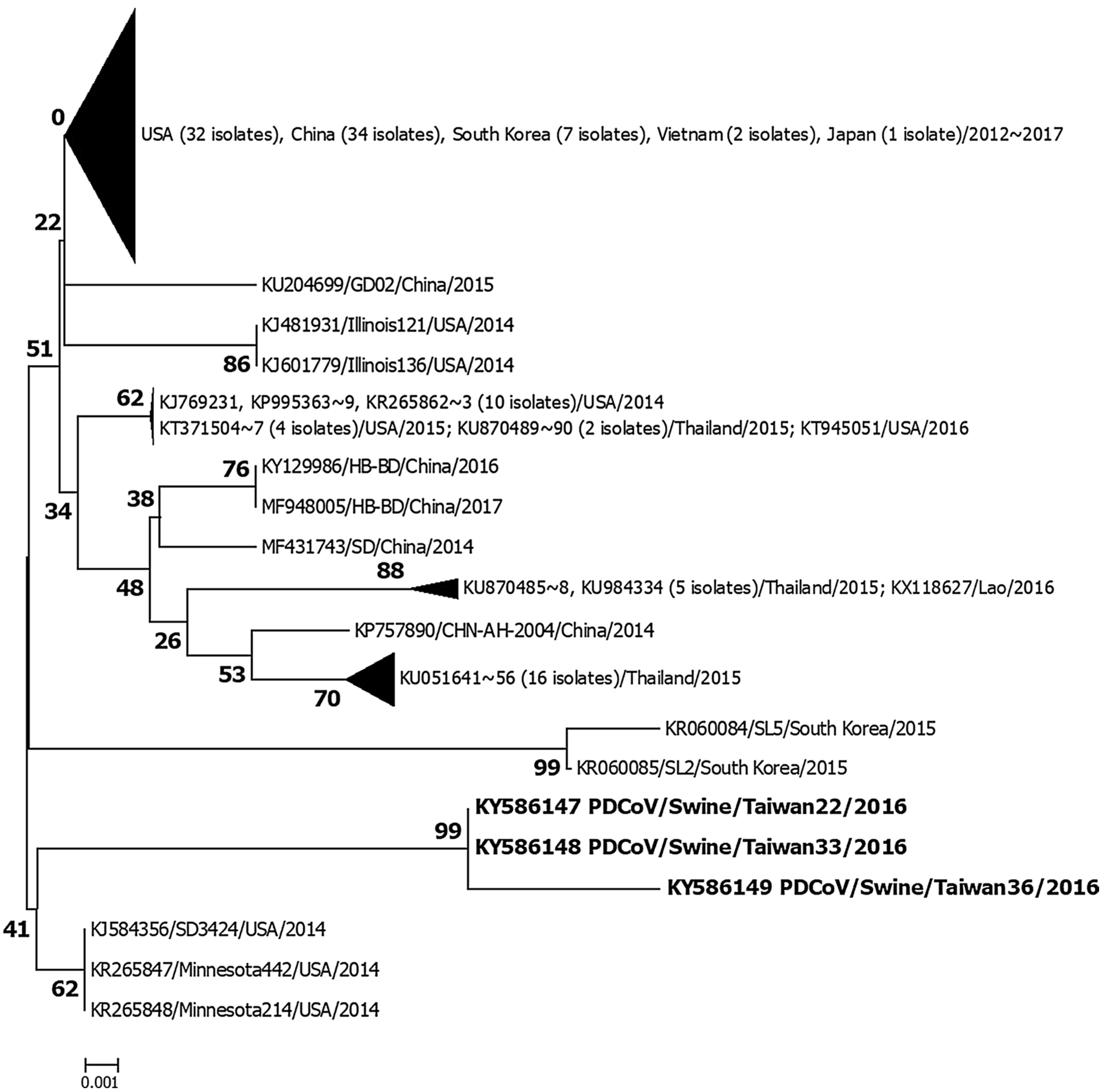

Fig. 2 Phylogenetic tree of the PDCoV-N polypeptide sequences constructed using the distance-based neighbor-joining method in MEGA7 [10]. All analyzed sequences obtained from GenBank were made available in August 2017 and are indicated by their accession number and/or strain name. The sequences obtained in this study are indicated by bold font. Bootstrap values were calculated with 1,000 replicates. The scale bar indicates the number of nucleotide substitutions per site

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                                     *                 #
    66           198           234          271   275
…IPPSFAFFYY…LPKGETISQ…MALAGHVPG…VKEGFPDYGRLKD…(TWN)
…IPPSYAFFYY…LPKGKTISQ…MALARHVPG…VKEGSPDYERLKD…(OTH)
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Fig. 3 Five major amino acid differences in the N proteins of PDCoVs—the 66th (F → Y), 198th (E → K), 234th (G → R/T), 271th (F → S), and 275th (G → E/D) amino acid residues—from Taiwan compared to those from other countries. The abbreviation "TWN" stands for Taiwan, and the abbreviation "OTH" stands for other countries. At the 234th amino acid residue (indicated by *) of the PDCoV-N protein, all three Taiwan isolates contained glycine, but all isolates from other countries contained arginine at this position, with the exception of one isolate from Thailand, which contained threonine. All three Taiwan isolates contained glycine at the 275th amino acid residue (indicated by #) of the PDCoV-N protein, but all isolates from other countries contained glutamic acid, except for KJ584356 (USA), KR265847 (USA), KR265848 (USA), and KR060084 (South Korea), which contained aspartic acid

To determine if PDCoV had already existed in Taiwan before 2016, the PDCoV-N protein was cloned, expressed, purified, and coated onto a 96-well microtiter plates for retrospective testing. Swine sera collected between 2011 and 2015 were tested for their reactivity to a PDCoV-N protein. The ELISA results showed that 279 of 1,039 (26.9%) sera were able to react with the PDCoV-N protein when [detected value > ($mean_{neg}$ +SD) × 2] was used as positive threshold, but only 48 of 1,039 (4.6%) sera were positive when [detected value > ($mean_{neg}$ +SD) × 3] was set as the threshold. The data indicated that PDCoV has existed in Taiwan since 2011. Similar ELISA tests were also used to investigate the presence of PDCoV in other studies [7, 11, 14, 15].

Based on our findings, we confirm that infection of PDCoV and its coinfection with PEDV in pigs have existed in Taiwan between 2011 and 2017. It is known that antibodies against PEDV, TGEV, or PDCoV provide no cross-protection against either of the other two coronaviruses [14, 16–19]. Coinfection with PEDV and PDCoV might explain why some efforts have been ineffective in the PEDV vaccination program. Therefore, a divalent vaccine to control PDCoV and PEDV is desperately needed.

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Compliance with ethical standards

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Conflict of interest Tien-Huan Hsu has received grants from the Bureau of Animal and Plant Health Inspection and Quarantine. All authors declare no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors. Rectal swabs were collected from clinically diarrheic piglets in pig farms in Taiwan, while swine sera were obtained from the Animal Disease Control Centers of various counties and the Animal Technology Institute Taiwan.

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