Original Article

Emergence of a classical variant of porcine epidemic diarrhea virus novel to Thailand responsible for the milder clinical disease in a herd previously infected with a pandemic variant

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Abstract

In 2014, porcine epidemic diarrhea (PED) outbreaks characterized by mild clinical disease and low pre-weaning mortality increased in frequency in Thailand. An outbreak investigation, based on the detection of the classical variant and the allowance of the herd owners, was conducted in 5 swine herds and 25 intestinal samples were submitted from herds with outbreaks of diarrhea for sequencing analysis. PEDV isolation and complete nucleotide sequences of the spike (S) glycoprotein were determined. The PEDV isolate EAS1 was identified. Phylogenetic analysis based on the full-length spike gene demonstrated that EAS1 belonged to the classical variant group with high nucleotide and amino acid identity (98.4-99.0% and 96.6-97.9%) with LZC and SM98, which are the Chinese and Korean classical variant PEDVs, respectively. The results suggest the emergence of a classical variant PEDV responsible for the mild clinical disease in Thailand. The introduction could have been due to the use of illegally imported modified live vaccines from other countries.

Keywords: Emergence, Novel, Porcine epidemic diarrhea virus, Spike gene, Thailand

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Introduction

Porcine epidemic diarrhea (PED), an enteric disease characterized by acute severe watery diarrhea and high mortality (Pensaert and De Bouck, 1978), is caused by the PED virus (PEDV), a virus in the family *Coronaviridae*. Two PEDV variants are recognized: classical and pandemic variants (Sun et al., 2015). The spike gene is a distinguishing feature. The pandemic variant contains two insertions of 4 (^{56}GENQ^{59}) and 1 (^{140}N) amino acids at positions 55-60 and 140, respectively, and a deletion of 2 amino acids (^{160}DG^{161}) at positions 160-161 (Li et al., 2012) and has been reported as an emerging variant worldwide (Li et al., 2012; Chen et al., 2014; Lee and Lee, 2014; Lin et al., 2014; Hanke et al., 2015; Masuda et al., 2015; Ojkic et al., 2015; Sun et al., 2015; Theuns et al., 2015; Vui et al., 2015).

PED first emerged in Thailand in late 2007 (Puranaveja et al., 2009). A herd in Nakhon Pathom, a province in the Western region of Thailand, reported a disease outbreak characterized by severe diarrhea and high mortality. The pandemic outbreak was then reported across Thailand. PEDV was detected and the variant responsible for the outbreaks and circulating in Thailand, was a pandemic variant closely related to Chinese and Korean isolates (Temeeyasen et al., 2014). Since then, PED has become endemic and causes sporadic outbreaks in which outbreaks of one or two times a year have been reported. Normally, repeated outbreaks of 4-6 months apart have been observed. However, in late 2013, repeated PED outbreaks with a shorter duration and a milder clinical disease were reported in several herds. It is worthy of note that outbreaks reoccurred within 2-3 months after a previous outbreak and displayed milder clinical disease compared to the earlier outbreaks.

It is unknown whether a novel variant of PEDV with milder clinical severity was introduced into Thailand or if previous isolates evolved until they were no longer recognized by the immune system. In order to identify the PEDV variants responsible for the recent outbreaks, intestinal samples from herds experiencing PED outbreaks with mild clinical disease were collected. PEDV was isolated and the full-length spike gene obtained from intestinal samples was characterized and compared to spike gene sequencing data previously reported.

Materials and Methods

***Herd selection and investigation*:** A disease investigation was conducted in swine herds that experienced PED outbreaks in Eastern and Western regions of Thailand from July 2014 to December 2014. Herd selection was based on three criteria. Firstly, the permission of the herd owners. Secondly, emphasis was placed on investigating swine herds that experienced a reoccurrent outbreak of PED with mild clinical disease. Mild clinical disease characterized by PED associated clinical signs included vomiting, diarrhea and dehydration but mortality was low compared to PED previously experienced. Low mortality was approximately 30-50%. Finally, the availability of PEDV sequencing data from previous outbreaks. The investigated herds were required to have PEDV sequencing data from previous PED outbreak to compare the genetic difference between the PEDV responsible for previous and current outbreaks. With these three criteria, only five herds were selected for further investigation. Face-to-face interviews were conducted at the farm. In the interview, information based on herd size, production type, PEDV vaccination use, killed (KV) or modified live virus (MLV) vaccine, how long had PEDV vaccines been used, frequency of repeated PEDV outbreaks and pre-weaning mortality following the current outbreak was collected.

***Source of specimens and PEDV isolation*:** Twenty-five intestinal samples were collected from 3-4-day-old pigs displaying severe watery diarrhea from 5 affected pig farms. The intestinal samples were submitted to the laboratory of Dr.Dachrit Nilubol from December 2013 to December 2014. The samples were assayed for the presence of swine coronaviruses including PEDV, transmissible gastroenteritis virus (TGEV) and porcine deltacoronavirus (PDCoV). Detection for PEDV and TGEV was performed following the previously described protocols using specific primers for spike (S) gene of PEDV (Park et al., 2007), and specific primers for N gene of TGEV (Kim et al., 2000). To screen for the presence of PDCoV, PCR amplification was performed on cDNA using specific primers for the N gene of PDCoV as previously described (Wang et al., 2014).

All intestinal samples positive for PEDV by PCR were subjected to PEDV isolation using the same protocol as the previous study (Temeeyasen et al., 2014). In brief, intestinal samples were minced into small pieces and suspended in 10 mL of phosphate buffer saline (PBS; 0.1 M, pH 7.2). Suspended samples were centrifuged at 4,800xg for 10 min. Supernatant was filtered through 0.22 μm filters and inoculated into a 25 cm^2 flask that previously estimated the fully confluent Vero cells. The inoculated flask was incubated at 37° C in 5% CO_2 for 1 hour, then replaced by 10 ml MEM 2% FBS and incubated again for a period of 2-3 days and daily observed for CPE. The CPE positive flask was frozen and thawed 2 times before being centrifuged at 2,500 rpm for 10 min to collect the supernatant and store at -80° C.

***Reverse transcription-polymerase chain reaction*:** Supernatant from PEDV isolation was subjected to RNA extraction using the Nucleospin®RNA Virus (Macherey-Nagel Inc., PA, USA) in accordance with the manufacturer's instructions and synthesized cDNA from the extracted RNA using M-MuLV Reverse Transcriptase (New England BioLabs Inc., MA, USA). Complete S genes PCR amplification was performed on the cDNA using previously described primers (Temeeyasen et al., 2014). *PCR products were visualized* by agarose *gel* electrophoresis.

***Cloning, plasmid purification and sequence determination*:** PCR products were cloned into plasmid vectors for the subsequent transformation of *Escherichia coli* cells using a commercial kit (pGEM-T® Easy Vector System I (Promega, WI USA), and the controls were included at all stages of cloning and transformation. Two to three bacterial transformant

colonies were randomly selected from each sample for plasmid purification using the Nucleospin® Plasmid kit (Macherey-Nagel Inc., PA, USA) and the selected colonies were grown in LB broth for 24 h and subjected to plasmid isolation and sequencing. Sequencing was performed by First BASE Laboratories Inc. (Selangor, Malaysia) using an ABI Prism 3730XL DNA sequencer.

***Sequence analyses*:** Nucleotide and deduced amino acid sequences were aligned using the CLUSTALW program (Thompson et al., 1994). A phylogenetic analysis was constructed based on complete S genes from this study and as previously reported. A midpoint-rooted neighbor-joining tree was generated based on the Kimura 2-parameter model using MEGA4 (Tamura et al., 2007). The robustness of the phylogenetic analysis and significance of the branch order were determined by bootstrap analysis with 1,000 replicates. The percentage of homology between the isolates at the nucleotide and amino acid levels was calculated.

Results

***Herd investigation*:** Five herds experiencing PEDV outbreaks were investigated. Two patterns of recurrent outbreak were observed according to the PEDV variants that were successfully isolated during the recurrent outbreaks. In two of the five herds, the recurrent PEDV outbreak occurred 16-24 weeks after the previous outbreaks and the causes of repeated PED outbreaks were pandemic variant. The mortality rates in these herds were 56.9 and 52.1% that were higher compared to those observed in herds in which the recurrent PEDV outbreaks had occurred within 3 months. Three of five herds had a recurrent outbreak within 12 week of the previous outbreak and the classical variant of PEDV were the cause of repeated outbreaks. The mortality rates in these 3 herds were 32.5%, 33.1% and 28.7%, respectively (Table 1).

***Phylogenetic analysis of complete spike gene*:** All 25 intestinal samples were PEDV-positive. The full-length spike gene of 25 PEDV isolates was sequenced. To investigate the heterogeneity, the 25 full-length spike genes (accession number KR610991-KR610993 and KR941552-KR941557) were aligned with 81 previously published PEDV spike genes, including the previously reported Thai PEDV. The phylogenetic analysis revealed that all PEDV isolates were grouped into 3 clusters: G1, G2 and G3 (Figure 1).

Table 1 Radiographic scoring system for evaluation of bone healing (Johnson et al., 1996b).

Herds	Geographic region	Herd size (sows)	Use of modified live PEDV vaccine	PEDV variants responsible for previous outbreak	PEDV variants responsible for recurrent outbreak	Duration from a previous outbreak	Pre-weaning mortality (%) during recurrent outbreak
DT	Central	1,300	Yes	Pandemic	Classical	8 weeks	32.5
JK	East	2,000	Yes	Pandemic	Classical	9 weeks	33.1
ET	East	2,000	No	Pandemic	Classical	12 weeks	28.7
SA	West	2,000	No	Pandemic	Pandemic	16 weeks	56.9
PR	West	1,200	No	Pandemic	Pandemic	24 weeks	52.1

The phylogenetic analysis demonstrated that Thai PEDV is presently categorized into two genetically distinct clusters: G1 and G3. Four Thai PEDVs isolated in 2014 included in G1 are novel to Thailand. Comparative analyses of the full-length spike genes demonstrated that the difference between both clusters lies in the characteristics of the spike gene. The PEDV isolates in G3 contained two insertions of 4 (^{56}GENQ^{59}) and 1 (^{140}N) amino acids at positions 55-60 and 140 of the S gene, respectively, and a deletion of 2 amino acids (^{160}DG^{161}) at positions 160-161. A previous study reported that Thai PEDV is a pandemic variant belonging only to G3. The emergence of PEDV in G1 suggests that this is a novel isolate to Thailand. The spike gene sequencing results showed that the newly isolated PEDV, Thai PEDV in G1, shared very high nucleotide and amino acid identity (98.4-99.0% and 96.6-97.9%, respectively) with LZC and SM98, which are the Chinese and Korean classical variant PEDVs, respectively. Vaccine virus was isolated from the vaccine vial and the sequence of the vaccine isolate was performed. The sequencing result demonstrated the vaccine isolate was similar to the classical variant PEDV isolates in this study (Figure 1). Nucleotide and amino acid identity between the vaccine isolate and the classical isolates in this study were 99.8% and 99.4%, respectively.

Discussion

Since its first emergence in Thailand in 2007, PED has become endemic in Thai swine farms and the PEDV responsible for disease outbreaks in Thailand has been a pandemic variant. A classical variant of PEDV had not been identified (Temeeyasen et al., 2014). However, in late 2013, several swine farms reported PED outbreaks with mild clinical disease and mainly low pre-weaning mortality. Intestinal samples were then submitted for further genetic characterization. The presence of other coronaviruses including TGE and PCDoV was ruled out due to the negative results of PCR. A disease investigation was conducted following the analysis of the sequencing

data. To compare the effect of repeated outbreaks caused by the classical variant and pandemic variant, the disease severity among these five investigated herds was determined using pre-weaning mortality. A previous study demonstrated the different pathogenicity of the classical variant and pandemic variant PEDV in the experimental challenge model in which piglets orally challenge with a S-INDEL variant strain, the classical variant PEDV, exhibited milder clinical outcomes when compared to that of a non-S-INDEL, the US-PEDV prototype that genetically related to the pandemic variant PEDV (Chen et al., 2016). The findings from the previous study are similar to the results of the pre-weaning mortality observed in the present investigation in which the mortality rate in herds repeated outbreaks with pandemic variant PEDV was high. In contrast, the herds with repeated outbreaks with classical variant PEDV showed milder clinical disease and a lower mortality rate. However, one might speculate that the milder clinical disease and lower mortality rate of the repeat outbreaks by classical variant PEDV could be due to the immune status of the herds. Due to one of three criteria used to recruit PED outbreak herds into the present investigation showed the genetic sequencing data of PEDV from previous outbreaks in which the PED-positive herds where the information from previous study indicated the pandemic variant PEDV was responsible for the outbreaks. The herd immunity was supposed to be specific immunity against pandemic variant PEDV. A previous study demonstrated the decline of PEDV-specific antibody level after 6 months post infection (Ouyang et al., 2015). It is suggested that the duration of the protection immunity should potentially last for 6 months. The findings are similar to the results of the present study in which the duration between the previous outbreak and the repeated outbreak by the pandemic variant PEDV varies between 4 to 6 months. In contrast, herds in this present study with repeated outbreaks by the classical variant PEDV, the duration between the previous outbreak and repeated outbreak was relatively shorter than 6 months. These results might be associated with the cross-protection activity between classical and pandemic variants of PEDV which can vary from partial to none. Therefore, the potential cross protection between the classical and pandemic variants should be further investigated.

The introduction of classical PEDV was investigated in these herds. The source of the introduction could be an illegally smuggled modified live PEDV vaccine (MLV) recently used as a pre-farrow vaccine in sow herds. In herds DT and JK, PED outbreaks were observed a few days following the administration of the MLV. A few bottles of the MLV were collected and the genetic characterization of the vaccine virus suggests that the vaccine virus and the virus causing the outbreak were genetically related. An additional investigation of the genetic sequence of this vaccine demonstrated that the full-length spike gene of the vaccine virus shares 99.8% and 99.4% similarity with the classical PEDV variant responsible for the outbreak in this study at the nucleotide and amino acid levels, respectively. In addition, the genetic characterization of the classical PEDV variant in this study shared 99.0% and 97.9% similarity at nucleotide and amino acid, respectively, with a vaccine variant from Korea (SM98). The emergence of a classical variant of PEDV related to modified live vaccine (MLV) was not surprising. Following PEDV outbreak, several swine herders have utilized the illegally imported MLV to control PEDV. The vaccination program includes pre-farrow vaccination with two doses of MLV. The vaccine virus in MLV may not be properly attenuated and could become infectious in vaccinated sows with the potential to shed the vaccine virus in the environment. The spill over from gestation unit to the farrowing unit might potentially occur through the worker and fomites which could carry the virus from unit to unit (Lowe et al., 2014). In addition, sows could potentially shed the vaccine virus to their piglets, which could lead to a severe disease outbreak. The route of excretion requires further investigation.

The detection of a vaccine-like variant in the herd ET is of interest. Even through, the MLV was not used in the herd the herd was located in a one of the highest densities of the swine producing areas in the Eastern region of Thailand. The virus could have been spread from some farms through airborne transmission which have evidence of 16 kilometers (Alonso et al., 2014), or from other route including transport trailer (Lowe et al., 2014) or dead hauling truck.

One controversy would be that the vaccine virus might have been in this region for quite some time. However, based on previous reports, this hypothesis is still debatable. The first report of PEDV emerging in Thailand suggested that, based on the partial spike sequencing data, the PEDV variant responsible for the outbreaks in Thailand was Chinese-like (Puranaveja et al., 2009). However, another investigation, that analyzed the nucleotide and amino acid sequence based on complete spike and ORF3 genes, found that the variants responsible in Thailand were all pandemic variants. In addition, the sequence analysis of ORF3 gene indicated none of the PEDV isolates was genetically related to MLV vaccines at that time (Temeeyasen et al., 2014). In addition, further evolution analyses including the identification of natural selection in different gene regions involving in the evolutionary process of PEDV suggesting that the emergence of classical PEDV variant in Thailand was due to the external introduction, rather than the continuous evolution in the population (Cheun-Arom et al., 2016).

In summary, the results of the study demonstrated the emergence of a novel PEDV in Thailand that is genetically related to the classical variant. The introduction, molecular epidemiology and cross protective immunity against the pandemic variant previously existing are of interest and require further investigation.

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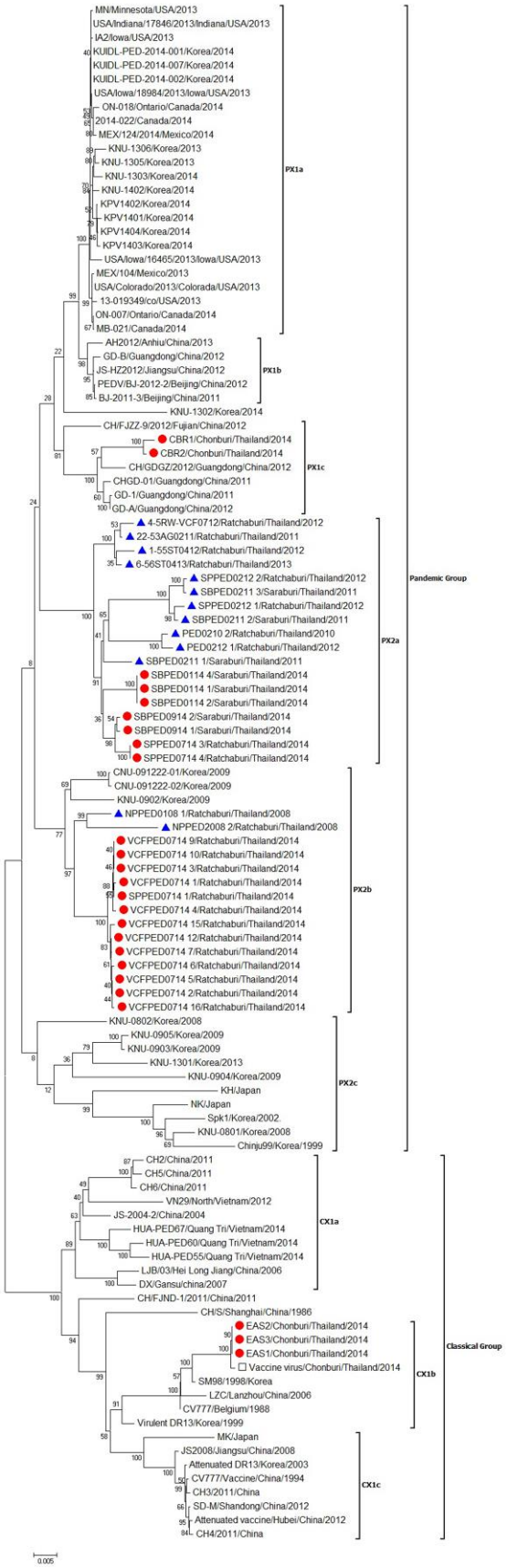

Figure 1 Phylogenetic analysis of porcine epidemic diarrhea virus (PEDV) isolates based on the nucleotide sequences of the complete S glycoprotein genes. The tree was constructed using the neighbor-joining method with the Kimura 2-parameter and bootstrap re-sampling (1,000 replications). Blue filled triangles indicate isolation before 2013. Red filled dots indicate isolation in 2014. Unfilled squares indicate isolation from vaccine.

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บทคัดย่อ

การอุบัติใหม่ของเชื้อไวรัส porcine epidemic diarrhea สายพันธุ์ดั้งเดิมในประเทศไทย ซึ่งก่อให้เกิดโรคชนิดอาการไม่รุนแรงในฝูงสุกรที่เคยมีการติดเชื้อสายพันธุ์ใหม่

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ในปี ค.ศ. 2014 พบว่าการระบาดของโรคท้องร่วงติดต่อในสุกร (โรคพีอีดี) ในฟาร์มสุกรของประเทศไทยมีลักษณะเปลี่ยนแปลงไป โดยมีความรุนแรงของโรคและอัตราการตายของลูกสุกรก่อนหย่านมน้อยลงเมื่อเปรียบเทียบกับการระบาดก่อนหน้านั้น แต่พบความถี่ในการระบาดสูงขึ้น จึงได้ทำการสืบสวนหาสาเหตุการระบาดของโรคโดยการตรวจสอบลักษณะทางพันธุกรรมของเชื้อไวรัสพีอีดีในฟาร์มที่แสดงอาการของโรคไม่รุนแรง โดยได้ดำเนินการสืบสวนโดยการรับตัวอย่างลำไส้จำนวน 25 ตัวอย่าง จากสุกรจำนวน 5 ฝูงที่มีการระบาดของโรคพีอีดี เพื่อทำการแยกเชื้อไวรัสพีอีดีและวิเคราะห์ลำดับสารพันธุกรรมของยีนสไปค์ชนิดทั้งสาย พบว่าเชื้อไวรัสพีอีดีไอโซเลต อีเอเอส 1 ที่ได้จากการเพาะแยกเชื้อ จัดอยู่ในกลุ่มสายพันธุ์ดั้งเดิม และมีลำดับนิวคลีโอไทด์และลำดับของกรดอะมิโนที่เหมือนกับไอโซเลต แอลแซสซี และไอโซเลต เอสเอ็ม 98 ซึ่งเป็นไวรัสสายพันธุ์ดั้งเดิมที่พบในประเทศจีนและประเทศเกาหลีใต้ถึง 98.4-99% และ 96.6-97.9% ตามลำดับ จากผลการศึกษานี้แสดงให้เห็นว่าการระบาดของโรคพีอีดีชนิดอาการไม่รุนแรงในประเทศไทยนั้น มาจากการระบาดของเชื้อไวรัสพีอีดีสายพันธุ์ดั้งเดิม ซึ่งสาเหตุของการระบาดเข้ามาของเชื้อดังกล่าว อาจมาจากการลักลอบนำวัคซีนเชื้อเป็นจากต่างประเทศเข้ามาใช้อย่างไม่เหมาะสม

คำสำคัญ: การอุบัติ ใหม่ ไวรัส porcine epidemic diarrhea ยีนสไปค์ ประเทศไทย

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