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BRIEF REPORT

Phylogenetic tracking of current porcine epidemic diarrhea virus (PEDV) strains in the Philippines

Rubigilda Paraguison-Alili[1] · **Clarissa Yvonne J. Domingo**[1]

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Abstract To trace the possible route of introduction of porcine epidemic diarrhea virus (PEDV), the phylogenetic relationships of PEDV strains in the regions of epidemicity in the Philippines to PEDV strains that are endemic in other countries were investigated. Partial nucleotide sequences of the S1 spike gene was determined from the PEDV-positive samples and compared with S1 sequences from other countries. Phylogenetic analysis indicated that PEDV strains in the Philippines segregate into two groups. Members of group 1 are related to strains from the USA, Taiwan, Japan and Canada, while those in group 2 are related to strains from China and Vietnam.

Introduction

Porcine epidemic diarrhea (PED) is an infectious enteric disease that poses a threat to the swine industry and is characterized by acute enteritis and diarrhea, with the infection causing more-severe disease in piglets. The causative agent of PED is porcine epidemic diarrhea virus (PEDV), which belongs to group 1a of the genus *Coronavirus*, family *Coronaviridae*, order *Nidovirales*. PEDV is an enveloped RNA virus with a single-stranded positive-sense RNA genome approximately 28 kb in size that contains six genes: replicase (Rep), spike (S), ORF3, envelope (E), membrane (M), and nucleoprotein (N). The full-length S gene is about 4.1 kb and consists of the S1 and S2 domains of approximately 2.2 and 1.9 kb in length, respectively. Since the receptor-binding sites and majority of the neutralization epitopes are located in the S1 portion, this region has been subjected to sequencing and molecular analysis to determine the genetic relatedness of different PEDV viruses [1–3]. The current study characterizes the spike S1 portion of the virus. The S protein has a crucial role in viral and cell fusion activity and in inducing a host immune response [4–6]. The spike protein makes up the surface projections of the virus and plays a major function in interaction with cell receptors [4]. The sequence of the full-length S gene, the S1 portion, or the S2 portion reflects the genetic diversity of the virus. The S gene, particularly the S1 portion, is a highly variable region and is appropriate for sequencing to study the genetic relatedness and molecular epidemiology of PEDV [1].

PED may occur year round, with peak incidence during the cold season, and it occurs in herds that have been exposed to the virus and have developed partial immunity. Pigs from 1 week of age are usually affected, and those that survive PED can be carriers for up to 104 days. Infected sows may be febrile, with occasional abortion, but they usually recover within 5-10 days. In the Philippines, the first case was reported in 1975 and was identified as a TGE-like virus on a farm of 800 sows in Pampanga. Recorded outbreaks occurred in January 2005 in Pampanga, Tarlac, Pangasinan, Batangas, Cavite, Laguna, Quezon and Negros Occidental. Sporadic and isolated outbreaks were recorded every year thereafter. Through 2016, outbreaks have been reported particularly in the provinces of Bulacan, Rizal, Pampanga, Tarlac, Pangasinan, Batangas, Cavite, Laguna, Bacolod and General Santos, with severe cases in adult animals. The mortality rates due to PED were as follows: Birth to 7 days old, 80 % to 100 %; 8 to 14 days old, 40 % to 50 %; 15 to 21 days

✉ Rubigilda Paraguison-Alili
rubigee@gmail.com

[1] College of Veterinary Science and Medicine, Central Luzon State University, 3120 Science City of Muñoz, Nueva Ecija, Philippines

old, 20 % to 25 %; 22 days or older, rare. The mortality decreases as age increases. Morbidity is high in growers and finishers, but mortality is low, and affected pigs may be stunted. In May to August 2010, an outbreak of PED occurred in the province of Batangas, killing 67 % (11,414 head) of 17,115 sick pigs, resulting in huge economic loss [7]. Linkages with swine farms in the provinces of Pampanga, Tarlac, Batangas and Agusan were established. Using data from swine farms in these provinces and diagnosis data from the College of Veterinary Science and Medicine in Central Luzon State University, the incidence is 67 % in Pampanga, 79 % in Tarlac, 61 % in Batangas, and 90 to 100 % in Agusan. The overall prevalence of PED is 65 %, 73 % on smallholding farms and 57.5 % on commercial farms.

Materials and methods

During the wet season of 2015, from July to October, swine farms from the provinces of Agusan, Batangas, Pampanga and Tarlac, which are known to experience PED outbreaks, were requested to provide clinical samples such as intestines, fecal samples, fecal swabs and environment swabs whenever there were reported cases. Selection of commercial and small swine farms was done in coordination with provincial veterinarians. Reports on cases of gastrointestinal infections with symptoms of diarrhea were obtained from veterinary offices. A list of affected farms was obtained and used in constructing the sampling frame. RNA was isolated using TRIzol reagent (Invitrogen USA) according to manufacturer's recommendations. Reverse transcription PCR was performed using a OneStep RT-PCR Kit (QIAGEN Germany). A pair of primers was designed to specifically amplify a portion of the S1 region: FS1M2 (GTC ATG GCA CTG ACG ATG ATG TTT C) and PEDS1R (CAG ATG TGT AAT AAA CAC CTG CCA A). Thermocyling conditions were as follows: 45 °C for 30 minutes and 95 °C for 10 minutes for the RT reaction, followed by 35 cycles of amplification at 94 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for 1 minute, with a final extension at 72 °C for 7 minutes. Good-quality RT-PCR products from samples that were positive for PEDV were selected and processed for DNA sequencing: Batangas, 1 sample; Tarlac, 5; Pampanga, 8 and Agusan, 2. A total of 16 PEDV-positive samples were processed for DNA sequencing of the 753-bp portion of the S1 gene. GenBank accession numbers for PEDV strains or clones from other countries are listed in Table 1. The partial S1 gene sequences of PEDV were aligned with those of strains from other countries, using ClustalW. A phylogenetic tree for the 753-bp gene segment was constructed using the maximum-likelihood method with the general time-reversible nucleotide substitution model. The confidence level for each branch was tested by the bootstrap method with 1,000 replicates. Phylogenetic and molecular evolutionary analysis was conducted using MEGA version 7.0.14 software [8].

Table 1 Reference sequences from NCBI GenBank, described by strain or clone names and accession numbers

Strain	Country	Accession no.
CH-CCC-2013	China	KT388421.1
Strain OKY-1/JPN/2014	Japan	LC063847.1
Strain CAN/Quebec334/2014	Canada	KR265831.1
Strain USA/Minnesota372/2014	USA	KR265843.1
Strain USA/Missouri270/2014	USA	KR265846.1
Strain TW-Pingtung-52	Taiwan	KP276252.1
436_2015_AUT	Austria	KT206206.1
L00721/GER/2014	Germany	LM645057.1
Strain CJ98	Korea	KJ857456.1
Clone VN/JFP1013_1/2013/Vinh An	Vietnam	KJ960178.1

Results and discussion

This is the first report of PEDV S1 gene sequences from the provincial PEDV hotspots of the Philippines, which include Batangas, Pampanga, Tarlac and Agusan. A phylogenetic tree was created by the maximum-likelihood method with 1,000 bootstrap replicates, using the MEGA7.0.14 software [8]. Interestingly, sequence analysis of the S1 gene from current PEDV strains from the Philippines revealed that these current strains cluster into two groups. Viruses in group 1 share common phylogenetic roots with strains from the USA, Taiwan, Japan and Canada, while those in group 2 were more closely related to strains from China and Vietnam. PEDV identified on swine farms in Pampanga, Tarlac (Central Luzon), Batangas (Southern Luzon), and Agusan (Mindanao) were clustered within group 1. Interestingly, other samples from Central Luzon, particularly Pampanga and Tarlac, clustered separately within group 2 (Fig. 1).

It has been reported that all US PEDV strains form a tight cluster and are most closely related to a strain isolated in 2012 in China (AH2012) [9], while Philippine strains are closely related to Vietnamese PEDVs in an ORF3 genetic tree [10]. In another study, comparative analysis of full-length sequences of the whole genome revealed that isolates from Germany show very high nucleotide sequence similarity to strain OH851, which was found in the United States in 2014 [11]. Phylogenetic analysis of the complete PEDV genome also indicated that 38 Japanese strains,

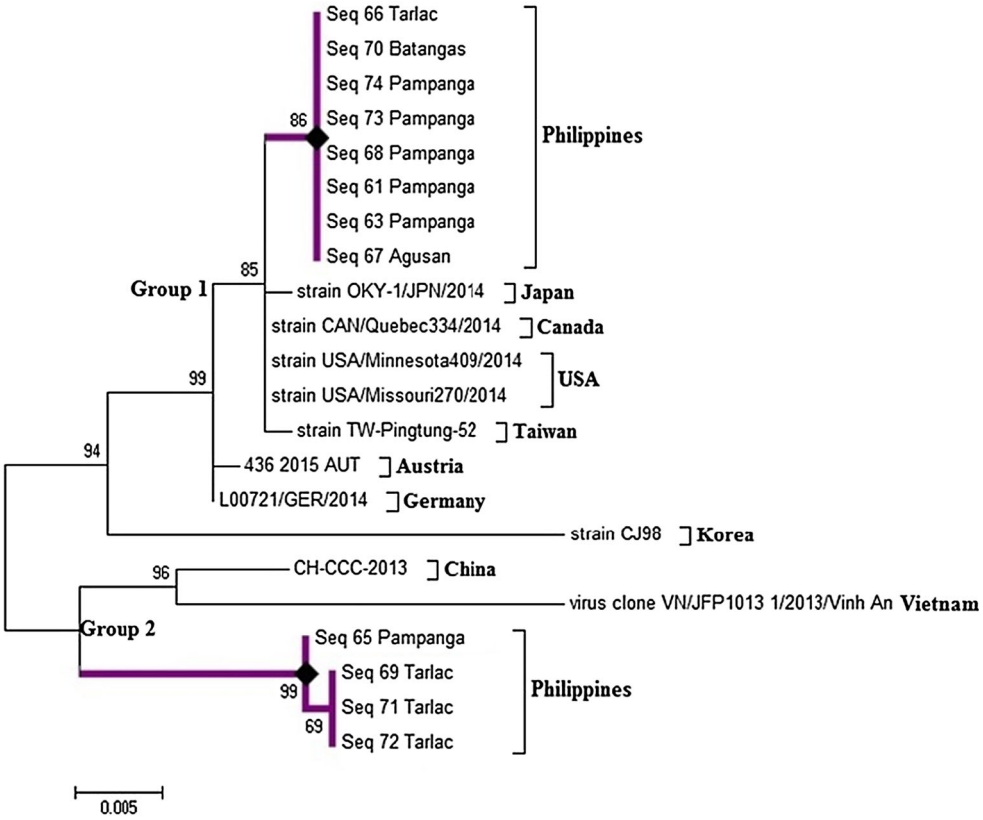

Fig. 1 Molecular phylogenetic analysis of PEDV in the Philippines. Phylogenetic tree showing PEDV strains from "hotspot" provinces of the Philippines along with the other strains from China, Taiwan, Vietnam, Korea, Japan, the USA, Austria and Germany. Phylogeny was based on partial spike S1 gene sequences. Evolutionary history was inferred using the maximum-likelihood method based on the Kimura 2-parameter model [16]. The initial tree for the heuristic search was generated automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach. Evolutionary analysis was conducted using MEGA7 [8]

including two novel PEDV variants, were closely related to strains that were widespread in the United States and Korea in 2013-2014 [12]. Moreover, sequencing and phylogenetic analysis of the spike gene and ORF3 of PEDV revealed that the prevailing PEDV isolates in Japan had the greatest genetic similarity to US isolates [13]. In Taiwan, phylogenetic analysis of the S gene showed that all PEDV strains from Taiwan were closely related to the non-S INDEL strains from the USA, Canada and China [14]. Taken together, recent reports are consistent with our findings that the Philippine PEDV strains share common ancestors with those from the USA, Taiwan, Japan and Canada (group 1) as well as those from China and Vietnam (group 2).

PEDV is present in many countries in Asia and has been present in Europe since the 1970s. It was first discovered in Europe, but it has become increasingly problematic in Asian countries such as Korea, China, Japan, the Philippines, and Thailand. It has also spread to North America and was discovered in 2013 in Indiana, USA [14], and in Canada in the winter of 2014. In January 2014, a new variant strain of PEDV with three deletions, one insertion, and several mutations in the spike 1 region was identified in Ohio by the Animal Disease Diagnostic Lab of the Ohio Department of Agriculture [15].

Findings presented in this paper provide important information regarding the route of introduction of PEDV in the Philippines and provide linkages to other countries. The emergence of different PEDV strains can occur via introduction of the virus from overseas, since this virus is versatile and can be easily spread. Our study also detected PEDV contaminants from inanimate objects such as swabs of floor pens and farrowing crates, fan blades of tunnel vent farms, raw feed ingredients, and inner surfaces of empty feed sacks. Hence, proactive disease control measures should include the identification of the source of imported goods for pigs, which may require thorough biosecurity procedures to limit the spread of the agent and the outbreak.

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Compliance with ethical standards

Conflict of interest The authors whose names are listed below certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria, educational grants, participation in speakers' bureaus, membership,

employment, consultancies, stock ownership, or other equity interest, and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript. We have no conflict of interest.

Statement of human rights All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Statement on the welfare of animals All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Informed consent Informed consent was obtained from all individual participants included in the study.

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