PEDV MONITORING USING ORAL FLUID, FECES AND SURFACE SWAB SAMPLES AFTER THE PED OUTBREAK

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การตรวจติดตามเชื้อไวรัสพีอีดีหลังการระบาดด้วยตัวอย่างของเหลวในช่องปาก ตัวอย่างมูลสุกรและตัวอย่างพื้นผิวในฟาร์ม

นางบุษยมาศ จันทราสกุล

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By	Mrs. Busayamas Jantrasakul
Field of Study	Veterinary Science and technology
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บุษยมาศ จันทราสกุล : การตรวจติดตามเชื้อไวรัสพีอีดีหลังการระบาดด้วยตัวอย่างของเหลวในช่องปาก ตัวอย่างมูลสุกรและตัวอย่างพื้นผิวในฟาร์ม. (PEDV MONITORING USING ORAL FLUID, FECES AND SURFACE SWAB SAMPLES AFTER THE PED OUTBREAK) อ.ที่ปรึกษาหลัก : ศ. ดร.รุ่งโรจน์ ธนาวงษ์นุเวช

ตัวอย่างทั้งหมดจำนวน 220 กลุ่มตัวอย่าง มาจากการเก็บทั้งหมด 3 ครั้ง หลังจากมีการสับไส้เพื่อให้แม่สุกรมีภูมิคุ้มกันครั้งแรกเริ่มที่ 1 เดือนหลังโรคพีอีดีระบาดมีจำนวน 80 กลุ่มตัวอย่าง เป็นตัวอย่างของเหลวในช่องปาก 10 กลุ่มตัวอย่าง ตัวอย่างมูลสุกร 30 กลุ่มตัวอย่าง และตัวอย่างพื้นผิว 40 กลุ่มตัวอย่าง ครั้งที่ 2 ทำการเก็บตัวอย่างที่ 2 เดือนหลังโรคพีอีดีระบาด มีจำนวน 85 กลุ่มตัวอย่าง เป็นตัวอย่างของเหลวในช่องปาก 10 กลุ่มตัวอย่าง ตัวอย่างมูลสุกร 30 กลุ่มตัวอย่างและตัวอย่างพื้นผิว 45 กลุ่มตัวอย่าง และครั้งที่ 3 ทำการเก็บตัวอย่างที่ 8 เดือนหลังโรคพีอีดีระบาด มีจำนวนตัวอย่าง 55 กลุ่มตัวอย่าง เป็นตัวอย่างของเหลวในช่องปาก 5 กลุ่มตัวอย่าง ตัวอย่างมูลสุกร 15 กลุ่มตัวอย่าง และ ตัวอย่างพื้นผิว 35 กลุ่มตัวอย่าง ทุกตัวอย่างถูกเก็บจากฟาร์มสุกรแห่งหนึ่งในจังหวัดชลบุรี ประเทศไทย ระหว่างเดือนกรกฎาคมปี 2560 จนถึงเดือนกุมภาพันธ์ปี 2561 จุดประสงค์ของการศึกษาในครั้งนี้เพื่อตรวจติดตามการคงเหลืออยู่ของเชื้อไวรัสพีอีดีภายในฟาร์มหลังเกิดการระบาดของโรคพีอีดี ด้วยการเก็บตัวอย่างของเหลวในช่องปาก ตัวอย่างมูลสุกรและตัวอย่างพื้นผิว จากนั้นทำการประเมินชนิดตัวอย่างที่สะดวกต่อการใช้ตรวจติดตามโรคพีอีดีหลังการระบาด จากตัวอย่างดังกล่าวทั้งหมดพบผลบวกทั้งสิ้น 20 ตัวอย่าง (20/220, 9%) ในการเก็บตัวอย่างครั้งที่ 1 พบการคงอยู่ของเชื้อไวรัสพีอีดี (2/80, 2.5%) ในมูลแม่สุกรสาวหลังคลอด 3 สัปดาห์และในมูลสุกรนางหลังคลอด 3 สัปดาห์ และพบการคงอยู่ของเชื้อไวรัสพีอีดีมากที่สุดในการเก็บตัวอย่างครั้งที่ 2 (18/85, 21.17%) โดยพบในตัวอย่างมูลสุกรแม่นางอุ้มท้องและมูลสุกรแม่นางหลังคลอด 1 สัปดาห์อย่างละ 1 ตัวอย่างและพบการคงเหลือเชื้อไวรัสพีอีดีในตัวอย่างพื้นผิวเล้าคลอดว่าง 1 ตัวอย่าง คอกคลอดสุกรสาวหลังคลอด 1 สัปดาห์ 5 ตัวอย่าง และสุกรนางหลังคลอด 1 สัปดาห์ 5 ตัวอย่าง คอกคลอดสุกรสาวหลังคลอด 3 สัปดาห์ 3 ตัวอย่างและสุกรนางหลังคลอด 3 สัปดาห์ 2 ตัวอย่าง และไม่พบการคงเหลือของเชื้อไวรัสพีอีดีในการเก็บตัวอย่างครั้งที่ 3 นอกจากนี้ยังไม่พบผลบวกจากการเก็บตัวอย่างด้วยของเหลวในช่องปาก จากผลการศึกษาทั้งหมดพบว่าภายหลังจากการระบาดของโรคพีอีดีที่ใช้การสับไส้เพื่อกระตุ้นการสร้างภูมิคุ้มกัน สามารถพบการคงเหลือของเชื้อไวรัสพีอีดีได้ในมูลสุกรและวัสดุอุปกรณ์ในฟาร์มได้ถึง 2 เดือนหลังการระบาด และตัวอย่างมูลสุกรและตัวอย่างพื้นผิวสามารถใช้ในการตรวจติดตามโรคพีอีดีหลังการระบาดได้ดี ดังนั้นการนำผลดังกล่าวไปเน้นการจัดการเฝ้าระวังการคงอยู่ของเชื้อจะเป็นหนึ่งในวิธีช่วยลดปัญหาการระบาดซ้ำภายในฟาร์มได้

สาขาวิชา วิทยาศาสตร์ทางการสัตวแพทย์และเทคโนโลยี ลายมือชื่อนิสิต .....

ปีการศึกษา 2561 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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KEYWORD: Fecal sample Monitoring Oral fluid sample Porcine epidemic diarrhea virus Surface swab sample

Busayamas Jantrasakul : PEDV MONITORING USING ORAL FLUID, FECES AND SURFACE SWAB SAMPLES AFTER THE PED OUTBREAK . Advisor: Prof. ROONGROJE THANAWONGNUWECH, Ph.D.

A total of 220 pooled samples from 3 collection times were conducted. Eighty pooled samples from the first collection at 1 month after PED outbreak (1MAO) included 10 pooled oral fluid samples, 30 pooled fecal samples and 40 pooled surface swab samples. Eighty-five pooled samples from the second collection at 2MAO included 10 pooled oral fluid samples, 30 pooled fecal samples and 45 pooled surface swab samples. Fifty-five pooled samples from the third collection at 8MAO included 5 pooled oral fluid samples, 15 pooled fecal samples and 35 pooled surface swab samples. All samples collected from the PED-affected swine farm in Chonburi province, Thailand between July 2017 to February 2018. The objective of this study was to evaluate the existence of PEDV residues in pigs and fomites in the affected swine farm after the PED outbreak and gut feedback protocol was implemented. Oral fluid, fecal and surface swab samples were collected to monitor the existence of the residue virus after the outbreak when practicing gut feedback protocol. The results showed that all pooled samples were positive to PEDV for 20 pooled samples (20/220, 9%). The samples at first collection (1MAO) were positive to PEDV (2/80, 2.5%) from a primiparous sow pen after 3 weeks farrowing and a multiparous sow pen after 3 weeks farrowing. The highest prevalence was found in the second sample collection (2MAO) and positive to PEDV (18/85, 21.17%) by 2 positive feces samples from multiparous gestated sows and multiparous sow after 1 week farrowing and 16 positive samples of surface swab samples from 1 positive sample from empty farrowed barn, 5 positive samples from primiparous sow pen after 1 week farrowing, 5 positive sample from multiparous sow pen after 1 week farrowing, 3 positive sample from primiparous sow pen after 3 week farrowing, 2 positive sample from multiparous sow pen after 3 week farrowing and no positive samples were found at the third sample collection (8 MAO). There have no any positive oral fluid samples from all samples. These findings demonstrated that fecal sample and surface swab sample could be used for PEDV detection after the outbreak up to 2 months when using gut feedback protocol. Viral eradication on the surface areas of the affected farm and PEDV monitoring from the fecal samples could be the effective tools for the prevention and control strategy.

Field of Study:	Veterinary Science and technology	Student's Signature .....
Academic Year:	2018	Advisor's Signature .....

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Busayamas Jantrasakul

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AR	Abortion rate
BA	Born alive
bp	base pair (s)
BW	Birth weight
CT	Cycle threshold
e.g.	exampla gratia, for example
ELISA	Emzyme-linked immunosorbent assays
EM	Electron microscopy
et al.	et alii, and others
F	Forward
FR	Farrowing rate
g	gravity
g	gram (s)
h	Hour (s)
IFA	Indirect fluorescent antibody test
IFT	Immunofluorescence test
IgA	Immunoglobulin A

IHC	Immunohistochemistry
Kg	Kilogram (s)
L	Liter (s)
m	Metre (s)
M	Molar (s)
MAO	Month after PED outbreak
mg	milligram (s)
min	minute (s)
ml	milliliter (s)
mm	Millimetre (s)
No.	number
ORF	Open reading frame
P>0	Multiparous
P0	Primiparpous
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PED	Porcine epidemic diarrhea
PEDV	Porcine epidemic diarrhea virus
pH	The negative logarithm of hydrogen ion concentration
R	Reverse

RNA	Ribonucleic acid
rpm	round per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
S	Spike
SB	Percentage of stillbirth piglets per litter
SDPP	Spray-dried porcine plasma
sec	second (s)
TAE	Tris-Acetate-EDTA
TB	World Organization for Animal Health
TCID	Polymerase Chain Reaction
TGEV	The negative logarithm of hydrogen ion concentration
USA	The United state of America
$	US dollar (s)
%	Percentage
℃	degree Celsius
µg	microgram (s)
µl	microliter (s)
µm	Micrometer (s)

CHAPTER I

INTRODUCTION

Porcine epidemic diarrhea (PED) is an emerging and re-emerging epizootic disease in the swine industry, recently. PED virus (PEDV) was described for the first time in Europe in the 1970s and has been persisting in Europe with sporadic cases until the late 1990's. Out-breaks of PED have also been described in Asia in the 1980s and to the North American countries in 2013 (Jung and Saif, 2015). PEDV can cause very huge economic losses due to its highly infectivity and rapidly spreading throughout the intensive swine industry by a severe, profuse watery diarrhea with or without vomiting and dehydration of all ages in swine and particularly dead in neonatal piglets up to 100% (Wang et al., 2016). The outbreak in new area with no immunological protection in piglets like in North America in 2013 have 100% loss of neonatal piglets and estimated the economic PEDV losses at farm level approximately $300,000 for a 700-sow farrow-to-finishing herd and the second outbreak might loss up to 50% pre-weaning mortality rate for 5 weeks (Weng et al., 2016). The susceptible pigs can infect with PEDV by direct or indirect fecal-oral route. PEDV can be introduced into the naïve herds by import the infected pigs, contaminated materials (fomites, feed ingredients, food, transport vehicles) and by contaminated people (Lee, 2015). PEDV transmission through contaminated milk from dam to piglets can also occur but vertical transmission of PEDV through semen has never been shown (Gallien et al., 2019). PEDV transmission through contaminated airborne transmission of the virus was also shown (Alonso et al., 2014). PEDV transmission through contaminated feed is one of important sources of PEDV. Previous study report PED contaminated feed and spray-dried porcine plasma (SDPP) used as a feed supplement were the source of the introduction of PEDV into Canada

(Pasick et al., 2014). Transportation is the major risk that can spread PEDV across the region (Lowe et al., 2014).

After the initial outbreak, the virus can survive up to 9 months in the infected manure storages in the farm (Tun et al., 2016). Although the residue level of virus in the environment is low, it can cause sporadic outbreaks within herd.

Therefore, the appropriated disease prevention and control methods including rapid and accurate diagnosis, careful treatment, biosecurity protocol, and husbandry management are of importance to overcome this problem.

Because of no specific effective treatment for PED, PED control and elimination measures aim to prevent the reinfection of PED in herds through the eradication of the virus and immunization of the breeding animals (Weng et al., 2016). Previous studies demonstrated that PEDV monitoring was focused on the immunity after the outbreak under field conditions. Lack of the information of the PEDV residues remaining in the swine farms after the outbreaks is still problematic.

Normally, after facing the PED outbreak, gut feedback procedure is implemented for inducing homogeneous PED herd immunity by using feces and/or chopped intestines from live infected neonatal piglets feeding to all sows and gilts for a period of 1–4 days (Gillespie et al., 2018). This protocol increases the virus load and immunity simultaneously in the farm and more importantly, this strategy effectively reduces the chance of PEDV perpetuation in successive batches of weaned piglets. After the feedback protocol, temporary closure of the herd like stopping animal introductions for a period of 4 months helps in building-up whole herd immunity. Intensive disinfection and biosecurity procedure have been used for PEDV eradication but PEDV residues remaining in the farms are needed to be monitored for checking or preventing the recurrent of PED outbreaks in the farms.

Therefore, the study objective was to evaluate the PEDV status from the remaining of viral residue and sample types of a PEDV positive herd. The investigation would provide benefit information needed for future PED management after facing with the PED outbreak.

Objectives of study

1. To evaluate the existence of PEDV residues in pigs and fomites in the affected swine farm after the outbreak when applying the gut feedback protocol by using oral fluid, fecal and surface swab samples for further management in the affected farm.
2. To identify proper sampling type for monitoring PEDV residues after the outbreak.

Questions of study

1. Can oral fluid, fecal and surface swab samples be the monitoring samples of choice for PEDV status after the PED outbreak?
2. Where could the PEDV residues be found in the PED positive farm?

CHAPTER II

LITERATURE REVIEW

Porcine epidemic diarrhea virus (PEDV) is a large-enveloped RNA virus belong to genus Alphacoronavirus in the Coronavirinae subfamily within the Coronaviridae family in the order Nidovirales (Gillam and Zhang, 2018). It is a highly contagious virus with rapid spread when introduced into the farms or the countries. High infectivity with low dose (10ml of inoculum with the titer of 0.0056 TCID50/ml (Ct>45) was able to cause infection in 25% of the 5 days old naïve inoculated pigs (Thomas et al., 2015). The incubation period is approximately 2 days. PEDV destroys villous enterocytes causing shortening of villi and thinning of intestinal walls leading to diarrhea. PED can differentiate from a related enteric coronavirus, the transmissible gastroenteritis virus (TGEV), where virus destruction of mature enterocytes leads to a marked reduction in their enzymatic activity, and disruption of digestion and cellular transport of nutrients and electrolytes resulting in malabsorptive diarrhea in piglets. Clinical signs in nursery to grower pigs show varied morbidity and mortality including depression, anorexia, diarrhea, and affecting the growth performances. Mild morbidity is found in boars or sows. PEDV can cause disease outbreak within 4-5 days and spread to other farms and regions via animal movement and contaminated fomites. Shedding times can be up to 6 days in 3-day-old piglets, 9 days in 2-week-old pigs, and 3 days in late-term pregnant sows (Song et al., 2005). Previous study showed that PEDV genetic material could be detected in the air up to 10 miles although the infectivity could not be shown (Alonso et al., 2014). Specific treatments are not available but symptomatic treatment of diarrhea is recommended in growing pigs depending on the farm veterinarians and owners if they want to eradicate or be with the PEDV (Lee, 2015). Vaccine is a tool for PED control and eradication during the outbreak by maintaining PEDV-specific neutralizing antibodies both in serum and

colostrum. While the piglets protection depend on acquired secretory IgA on intestinal mucosa (Langel et al., 2016), vaccine efficiency is still questionable in sows. Gut feedback is a common solution method but the successful rate varies by the amount of PEDV used, farm health status or even the gut feedback protocols used. Various PEDV diagnostic test includes immunofluorescencetest (IFT), immunohistochemistry (IHC), enzyme-linked immunosorbant assays (ELISA), polymerase chain reaction (PCR), indirect fluorescent antibody test (IFA), in situ hybridization, and electron microscopy (EM) from available samples; Feces, serum, fecal swab, intestine, oral fluid, environment sample and feed (Pospischil et al., 2002). Thus, monitoring is the way to know the farm status after the outbreak and a quality of gut feedback protocol done previously. The monitoring technique could be by checking the herd immune status on sows or surveying the viral residues and measuring the risk and progress of the disease.

CHAPTER III

MATERIALS AND METHODS

This experiment was divided into three phases, including Phase 1: Farm selection, sample collection, and data collection, Phase 2: Sample testing and Phase 3: Data analysis.

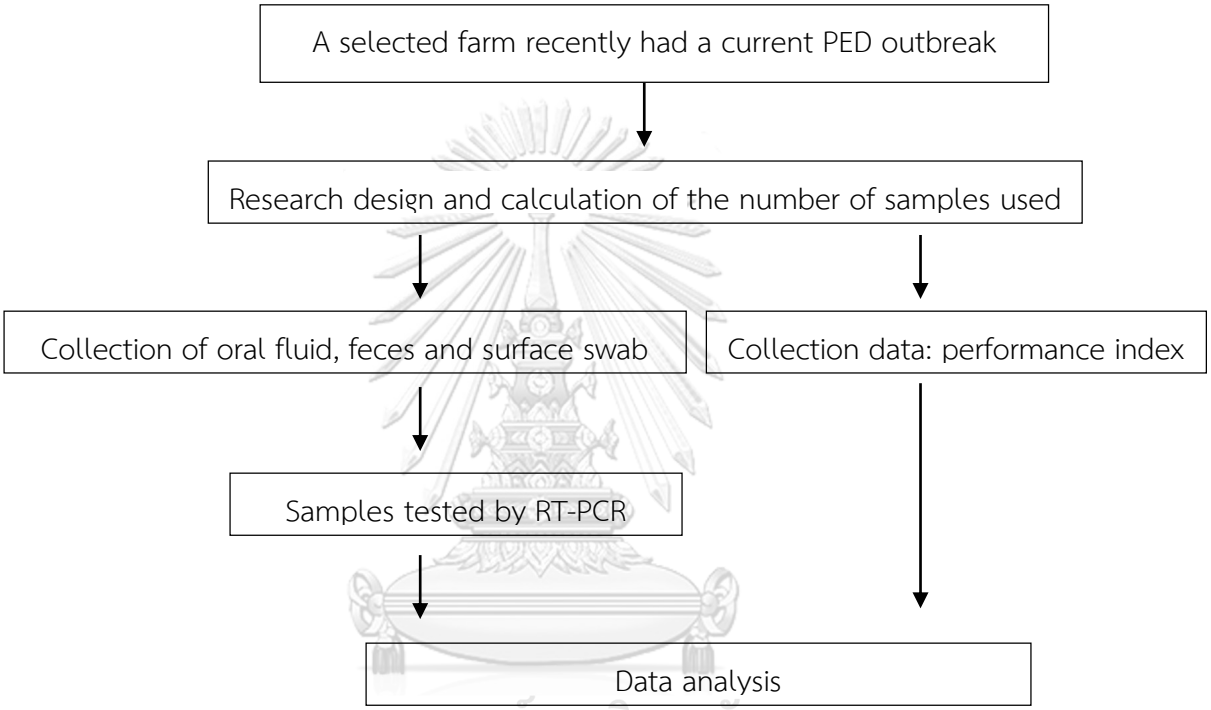

Figure 1 Flow chart of the experiments

Phase I: Farm selection, Sample collection, and Data collection

1. Farm selection

The criteria of a swine farm selected must have the recent PED outbreak and use gut feedback protocol for stabilizing the herd immunity. This selected swine farm located in Chonburi province, Eastern part of Thailand. The farm is a 700-sow herd with batch farrowing production system.

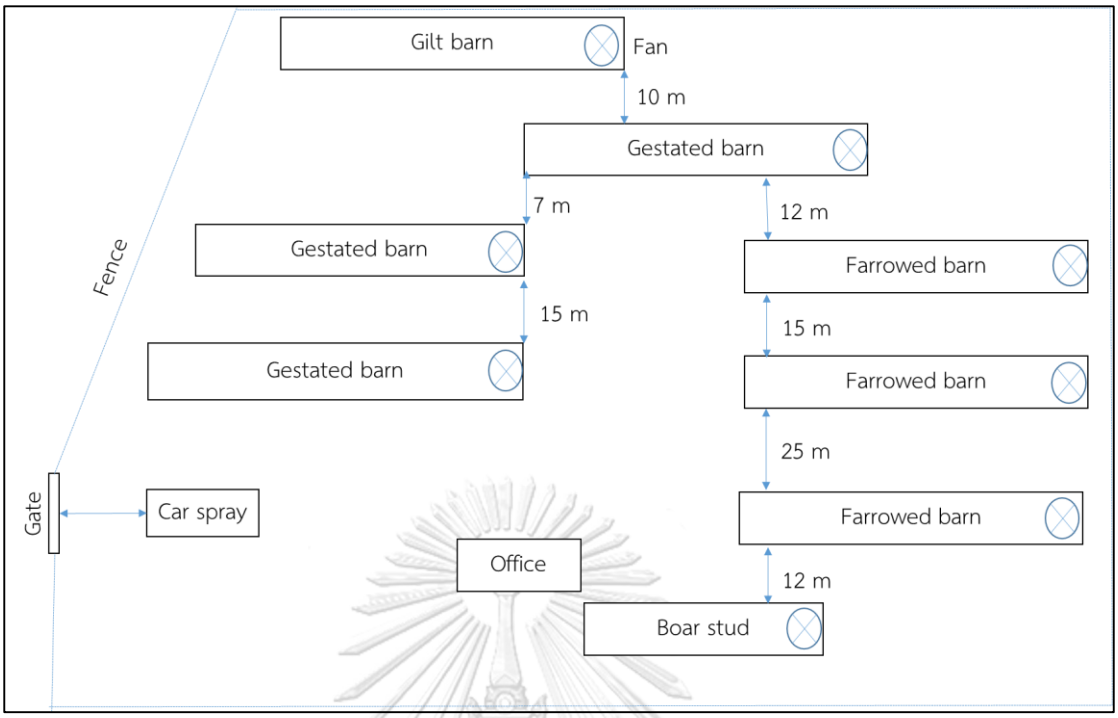

Figure 2 Farm layout

2. Sample collection

From around 200 farrowed sow, 600 gestated sows and 25 gilts, we randomized sampling a total of 1100 samples (220 pooled samples) were collected 3 times during the study (Table 1). The first collection time was at 1 month after the PED outbreak (MAO) (n=400, 80 pooled samples) containing oral fluid (n=50, 10 pooled samples; 5 each from gilt and primiparous gestated sow), fecal swab (n=150, 30 pooled samples; 5 each from primiparous gestated sow, multiparous gestated sow, primiparous farrowed sow at 1st week after farrow, primiparous farrowed sow at 3rd week after farrow, multiparous farrowed sow at 1st week after farrow and multiparous farrowed sow at 3rd week after farrow), and surface swab (n=200, 40 pooled samples; 5 each from empty gilt barn, empty farrowed barn, farrowed crates of primiparous farrowed sow at 1st week after farrow, farrowed crates of primiparous farrowed sow at 3rd week after farrow, farrowed crates of multiparous farrowed sow at 1st week after farrow, farrowed crates of multiparous farrowed sow at 3rd week

after farrow, boots and bedding). The second collection time was at 2MAO (n=425, 85 pooled samples) containing oral fluid (n=50, 10 pooled samples; 5 each from primiparous gestated sow and multiparous gestated sow), fecal swab (n=150, 30 pooled samples; 5 each from primiparous gestated sow, multiparous gestated sow, primiparous farrowed sow at 1st week after farrow, primiparous farrowed sow at 3rd week after farrow, multiparous farrowed sow at 1st week after farrow and multiparous farrowed sow at 3rd week after farrow), surface swab (n=225, 45 pooled samples; 5 each from empty gilt barn, empty gestated barn, empty farrowed barn, farrowed crates of primiparous farrowed sow at 1st week after farrow, farrowed crates of primiparous farrowed sow at 3rd week after farrow, farrowed crates of multiparous farrowed sow at 1st week after farrow, farrowed crates of multiparous farrowed sow at 3rd week after farrow, boots and bedding). The third collection time was at 8MAO (n=275, 55 pooled samples) composing of oral fluid (n=25, 5 pooled samples; from multiparous gestated sow), fecal swab (n=75, 15 pooled samples; 5 each from multiparous gestated sow, multiparous farrowed sow at 1st week after farrow and multiparous farrowed sow at 3rd week after farrow), surface swab (n=175, 35 pooled samples; 5 each from empty gilt barn, empty gestated barn, empty farrowed barn, farrowed crates of multiparous farrowed sow at 1st week after farrow, farrowed crates of multiparous farrowed sow at 3rd week after farrow, boots and bedding).

Table 1 Sample collection

Sample type	Collecting place	No.of samples (pooled)	1MAO (total 80)	2MAO (total 85)	8MAO (total 55)
Oral fluid sample	gilt	25(5)	✓	×	×
	pregnant gilt	25(5)	✓	✓	×
	multiparous gestated sow	25(5)	×	✓	✓
Feces sample	pregnant gilt	25(5)	✓	✓	×
	multiparous gestated sow	25(5)	✓	✓	✓
	primiparous sows after 1 week farrowing	25(5)	✓	✓	×
	primiparous sows after 3 week farrowing	25(5)	✓	✓	×
	multiparous sows after 1 week farrowing	25(5)	✓	✓	✓
	multiparous sows after 1 week farrowing	25(5)	✓	✓	✓
Surface swab sample	empty gilt barn floor	25(5)	✓	✓	✓
	empty gestated barn floor	25(5)	×	✓	✓
	empty farrowed barn floor	25(5)	✓	✓	✓
	primiparous sow pen after 1 week farrowing	25(5)	✓	✓	×
	primiparous sow pen after 3 week farrowing)	25(5)	✓	✓	×
	multiparous sow pen after 1 week farrowing	25(5)	✓	✓	✓
	multiparous sow pen after 3 week farrowing	25(5)	✓	✓	✓
	Cleaned boots	25(5)	✓	✓	✓
	Cleaned bedding	25(5)	✓	✓	✓

Sample collection was conducted using random sampling in multiple barns on a site to increase the probability of detection by collecting 5 samples for each type of pooled samples per units (Rotolo et al., 2017). Briefly, oral fluid samples were collected by hanging a cotton rope in each pen, with the end of the rope hanging at the height of the pigs' shoulder after chewing the rope for 20–30 min, the wet portion of the rope was put into a gallon size of single-use zip plastic bag and the rope will be squeezed for collecting of the fluid and then zipped, labelled and placed the bags on ice for transporting to the laboratory (Rotolo et al., 2017). Individual faecal sampling was done by covering the hand with the rubber gloves and collecting the faeces during defecation and, then put into the sealed plastic bag, labelled and placed in ice box (gloves must be changed in each pig). Surface swab sampling was done by using 10x10 cm of towels, soaked with 40 ml of PBS solution in 50 ml tubes. The towels were used to wipe 5 floor areas of 1 square meter before pooling into one sample in a 50 ml tube, labelled and kept in the ice box before delivery to the laboratory (Poonsuk et al., 2013).

A.

B.

C.

D.

Figure 3 Sample collection methods from swine farm (A) Oral fluid collection (B) Fecal sample collection (C) Surface swab sample collection (floor swab) (D) Surface swab sample collection (bedding swab)

3. Data collection

Performance index data were monitored including farrowing rate (FR), return rate (RR), abortion rate (AR), number of total piglets born per litter (TB), number of piglets born alive per litter (BA), percentage of stillbirth piglets per litter (SB), percentage of mummified fetuses per litter (MM), piglet's birth weight (BW), pre weaning mortality rate and total weaned pigs (Olanratmanee et al., 2010).

Phase II: Detection of PEDV (*S* gene and *ORF3* gene)

1. Sample preparation

PBS buffer was added to fecal sample before centrifuging with the rest of the samples (oral fluid, feces and surface swab samples) at 1,500×g for 15min to collect the supernatant fluids, individually. The supernatant fluids were stored at –80°C until tested.

2. RNA extraction

RNA was extracted from the supernatant using the Geneaid Viral nucleic acid extraction kit II (Geneaid Biotech. Ltd., Taipei, Taiwan). Briefly, 200 µl of the supernatant was transferred to a 1.5 ml microcentrifuge tube and 400 µl of VB Lysis Buffer was added. The mixture was mixed by vortex and incubated at room temperature for 10 min before adding 450 µl of AD Buffer to the sample lysate and shaking the tube vigorously. Six-hundred µl of the lysate mixture was then filled in a VB Column in a 2 ml collection tube and centrifuged at 14-16,000 x g for 1 min and repeat the procedure with the remaining mixture and discarded the 2 ml collection tube containing the flow-through. Adding 400 µl of W1 Buffer to the VB Column, centrifuging at 14-16,000 x g for 30 sec, discarding the flow-through, placing the VB

Column back in the 2 ml collection tube was done twice before placing the dried VB Column in a clean 1.5 ml microcentrifuge tube. Fifty µl of RNase-free Water was added to the center of the VB Column matrix and keep it stand for at least 3 min to ensure the RNase-free Water is absorbed by the matrix before centrifuging at 14-16,000 x g for 1 min to elute the purified nucleic acid.

3. Primers

Pairs of sense and antisense primers were aligned based on the nucleotide sequence of the spike, ORF3 genes from the GenBank database (National Center for Biotechnology Information, USA) (Sun et al., 2014) These primers were used to amplify the PEDV gene of interest. The primer sequences and other details are shown in Table 2.

Table 2 Primers used in RT-PCR for PEDV spike and ORF3 genes.

Primers	Nucleotides sequences (5'-3')	Size of product (bp)
S-F	TTCTGAGTCACGAACAGCCA	651
S-R	CATATGCAGCCTGCTCTGAA	
ORF3-F	TCCTAGACTTCAACCTTACG	833
ORF3-R	GGTGACAAGTGAAGCACAGA	

4. RT-PCR

RT-PCR was performed using The Invitrogen™ SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase, CA, USA). Primers S-F and S-R, ORF3-F and ORF3-R, as shown in Table 2, were used to amplify the corresponding partial S and ORF3 genes from PEDV, respectively. For RT-PCR, 3 µL of extracted viral

RNA was mixed with a reaction mixture containing 50 µL Buffer, 2X Reaction Mix 25 µL, 1 µL each specific primer, 3 µL RNA template, 2 µL SuperScript™ III RT/Platinum™ Taq Mix and 18 µL RNase Free water. The amplifications for the S, ORF3 genes were performed with reverse transcription at 55°C for 30 min and initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 15 sec, annealing at 55°C for 30 sec, and extension at 68°C for 1 min, and a final extension step of 68°C for 5 min. RT-PCR products were visualized via electrophoresis in a 1.5% agarose gel stained with Redsafe™ Nucleic Acid Staining Solution (iNtRon Biotechnology®, Seongnam, South Korea). A 5 µl of PCR product was electrophoresed by using 1.5% agarose gel electrophoresis (Vivantis®, Subang Jaya Malaysia) in 1×Tris-acetate/ EDTA (ethylene diamine teteraacetic acid) (1×TAE) buffer. The PCR product was then visualized under the UV light by Bio-Rad Gel-Documentation System (Bio-Rad Laboratories, USA).

5. Statistical Analysis

The descriptive statistics was conducted in this study and described the point of PEDV contamination and to which sample type are the most sensitive and practical for PED monitoring after the outbreak.

CHAPTER IV

RESULTS

1. Prevalence of PEDV

A total of 220 pooled samples were collected from the studied swine farm and found PEDV positive for 9% (20/220) (Table 3). The samples from the first collection (1MAO) were PEDV positive for 2.5% (2/80) only from the fecal samples. The highest prevalence was found in the second sample collection (2MAO) for 21.17% (18/85) ; 2 positive samples from feces and 16 positive samples from surface swab samples) and no PEDV positive sample was found at the third sample collection (8MAO).

Table 3 Prevalence of PEDV in the studied swine farm after the outbreak when using the gut feedback protocol, Chonburi, Thailand

Sample type	Collecting place	RT-PCR results			
		No.of Samples (pooled)	No. of positive (% of positive)		
			1MAO (total 80)	2MAO (total 85)	8MAO (total 55)
Oral fluid sample	gilt	5	0/5 (0%)	-	-
	pregnant gilt	5	0/5 (0%)	0/5 (0%)	-
	Multiparous gestated sow	5	-	0/5 (0%)	0/5 (0%)
Total			0/10 (0%)	0/10 (0%)	0/5 (0%)
Feces sample	pregnant gilt	5	0/5 (0%)	0/5 (0%)	-
	Multiparous gestated sow	5	0/5 (0%)	1/5 (20%)	0/5 (0%)
	primiparous sows after 1 week farrowing	5	0/5 (0%)	0/5 (0%)	-
	primiparous sows after 3 week farrowing	5	1/5 (20%)	0/5 (0%)	-
	multiparous sows after 1 week farrowing	5	0/5 (0%)	1/5 (20%)	0/5 (0%)
	multiparous sows after 3 week farrowing	5	1/5 (20%)	0/5 (0%)	0/5 (0%)

Sample type	Collecting place	RT-PCR results			
		No.of Samples (pooled)	No. of positive (% of positive)		
			1MAO (total 80)	1MAO (total 80)	1MAO (total 80)
Surface swab sample	empty gilt barn floor	5	0/5 (0%)	0/5 (0%)	0/5 (0%)
	empty gestated barn floor	5	-	0/5 (0%)	0/5 (0%)
	empty farrowed barn floor	5	0/5 (0%)	1/5 (20%)	0/5 (0%)
	primiparous sow pen after 1 week farrowing	5	0/5 (0%)	5/5 (100%)	-
	primiparous sow pen after 3 week farrowing	5	0/5 (0%)	3/5 (100%)	-
	multiparous sow pen after 1 week farrowing	5	0/5 (0%)	5/5 (75%)	0/5 (0%)
	multiparous sow pen after 3 week farrowing	5	0/5 (0%)	2/5 (40%)	0/5 (0%)
	Cleaned boots	5	0/5 (0%)	0/5 (0%)	0/5 (0%)
	Cleaned bedding	5	0/5 (0%)	0/5 (0%)	0/5 (0%)
Total			0/40 (0%)	16/45 (35.55%)	0/35 (0%)
Total			2/80 (2.5%)	18/85 (21.17%)	0/55 (0%)

Based on the results, no positive sample from oral fluid samples was found. Similar prevalence from feces sample at 1MAO and 2MAO and the farrowed barns floor were found to be the highest place having PEDV contamination from both 1MAO and 2MAO but found higher positive samples at 2MAO. No positive samples from cleaned boots and cleaned bedding and no positive results were found at 8MAO from all sample types.

Table 4 Details of positive fecal samples

Time	Collecting place	Gene of detection	
		Spike	ORF3
1MAO	primiparous sows after 3 week farrowing	-	+
	multiparous sows after 3 week farrowing	-	+
2MAO	multiparous gestated sow	-	+
	multiparous sows after 1 week farrowing	-	+

Table 5 Details of positive surface swab samples

Collecting place	Total sample (pooled)	No. of positive (% of positive)		Gene of detection	
				Spike	ORF3
empty farrowed barn floor	5 (cleaned area =2, Dirty area =3)	Dirty area #4 sample	1/3 (33.33%)	-	+
primiparous sow pen after 1 week farrowing	5 (cleaned area =2, Dirty area =3)	Cleaned area #1 sample	2/2 (100%)	+	+
		#2 sample		+	+
		Dirty area #3 sample	3/3 (100%)	+	+
		#4 sample		+	+
		#5 sample		-	+
primiparous sow pen after 3 week farrowing	5 (cleaned area =2, Dirty area =3)	Cleaned area #1 sample	1/2 (50%)	+	+
		Dirty area #3 sample	2/3 (66.67%)	-	+
		#4 sample		-	+
multiparous sow pen after 1 week farrowing	5 (cleaned area =2, Dirty area =3)	Cleaned area #1 sample	2/2 (100%)	+	+
		#2 sample		+	+
		Dirty area #3 sample	3/3 (100%)	+	+
		#4 sample		+	+
		#5 sample		+	+
multiparous sow pen after 3 week farrowing	5 (cleaned area =2, Dirty area =3)	Cleaned area #1 sample	1/2 (50%)	-	+
		Dirty area #5 sample	1/3 (33.33%)	+	+
Total 16 positive samples				11/16 (68.75%)	16/16 (100%)

Based on the results, all positive samples of fecal sample were only detected by ORF3 gene. And all positive surface swab samples can detect by ORF3 gene but only 68.75% can detect by S gene. For all positive samples can be detected by ORF3 but only 55% (11/20) can be detected by S gene.

2. The performance index data

The performance index data were monitored including the farrowing rate (FR), return rate (RR), abortion rate (AR), number of total piglets born per litter (TB), number of piglets born alive per litter (BA), percentage of stillbirth piglets per litter (SB), percentage of mummified fetus per litter (MM) and piglet's birth weight (BW), pre weaning mortality rate and total wean pigs (Olanratmanee et al., 2010) for 4 time points (average 5 months before the outbreak as the farm standard performance, at 1MAO, at 2MAO and at 8MAO). These indexes demonstrated the impact after PED outbreak.

A comparison of performance index between before and after the PED outbreak when using the gut feedback protocol showed that the farrowing rate had decreased from 79.74% to 75.20% at 1MAO, to 74.90% at 2MAO and to 73.80% at 8MAO. The return rate had changed from 3.33% to 6.80% at 1MAO, to 5% at 2MAO and to 3.2% at 8MAO. The abortion rate had changed from 2.40% to 3.20% at 1MAO, to 2.80% at 2MAO and to 3.00% at 8MAO. The total piglets born per liter had changed from 12.64 to 12.60 at 1MAO, to 12.30 at 2MAO and to 12.40 at 8MAO. The piglets born alive per litter had changed from 10.66 to 9.80 at 1MAO, to 10.40 at 2MAO and 8MAO. The percentage of stillbirth piglets per litter had changed from 13.42% to 19.20% at 1MAO, to 14.00% at 2MAO and to 14.40% at 8MAO. The percentage of mummified fetuses per litter had changed from 2.14% to 3.10% at 1MAO, to 1.80% at 2MAO and to 2.00% at 8MAO. The piglet's birth weight had changed from 1.68 to

1.70 at 1, 2 and 8MAO. The pre-weaning mortality rate had changed from 12.66% to 100% at 1MAO, to 50% at 2MAO and to 28.40% at 8MAO. The total wean pigs had changed from 1767.60 to 0 at 1MAO, to 960 at 2MAO and to 1489 at 8MAO.

Table 6 The performance index of the studied swine farm

Performance index	Data collection duration			
	Before the outbreak	1MAO	2MAO	8MAO
Farrowing rate (FR)	79.74	75.20	74.90	73.80
Return rate (RR)	3.33	6.80	5.0	3.20
Abortion rate (AR)	2.40	3.20	2.80	3.00
Total piglets born per litter (TB)	12.64	12.60	12.30	12.40
Piglets born alive per litter (BA)	10.66	9.80	10.40	10.40
Percentage of stillbirth piglets per litter (SB)	13.42	19.20	14.00	14.40
Percentage of mummified fetus per litter (MM)	2.14	3.10	1.80	2.00
Piglet's birth weight (BW)	1.68	1.70	1.70	1.70
Pre-weaning mortality rate	12.66	100	50	28.40
Total wean pigs	1767.60	0	960	1489

CHAPTER V

DISCUSSIONS

Porcine epidemic diarrhea virus (PEDV) is a highly contagious disease in swine. PEDV was described in 1971 in England and continued to spread and to persist as an endemic disease in the swine population of several countries. Many countries attempt to find the ways to control and eradicate this disease (Lee, 2015). However, in endemic areas or even in the affected swine farms after the PED outbreak, the strategies implementing to control the spreading of the virus are strict biosecurity and herd immunization (Kim et al., 2017). The gut feedback protocol is a routine method using the lives virus from infected piglets to immunize the whole sow herd (Song et al., 2015). However, the virus does have a chance to spread all over the farm and may persist in the environment as well. It should be noted that the gut feedback protocol must be done once for the homogenized herd immunization and followed by disinfection and cleaning up the farm environment to minimize sporadic shedding. The next step is focusing on the strict biosecurity for PEDV eradication and then, PEDV monitoring should be implement for detecting PEDV remaining in the farms.

Normally, the sow immunity is measured as the indicator for the success of the protocol used but, in this study, we monitored the viral residues from oral fluid, fecal swab and surface swab samples to evaluate the remaining of the virus in the studied farm. It should be noted that the studied farm had performed the close herd strategy after facing with the outbreak. Therefore, no sample from gilts at 2MAO and 8MAO and some first parity sows at 8MAO were obtained.

From the study, 2 genes (S gene and ORF3 gene) were used to increase the sensitivity of the findings. The reason of using S gene is that this site is critical for the viral entry and for inducing neutralizing antibodies (Jung and Saif, 2015). ORF 3 gene

is an important site using as a marker for attenuated and virulent strains and this site are conserved among strains from different temporal and geographical origins (Sun et al., 2014). We tested all samples twice for S gene and ORF3 gene and the positive samples at least from 1 gene was considered as a positive sample and we found all positive samples were from ORF3 gene and 55% (11/20) was from S gene.

One of the main findings of this study was the evidence of high contaminations of PEDV at 1MAO and at 2MAO. Some animals still showed diarrhea in new born piglets. Interestingly, surface swab samples were found positive at 2MAO higher than that of at 1MAO. This scenario could be explained that at 1MAO, the surface swab samples were done after the cleaning and disinfecting but at 2MAO and 8MAO, the surface swab samples were done for both clean and dirty areas. The remaining of PEDV in the environment at 2MAO showed that farm biosecurity and disinfection was not good enough. It should be noted that cleaning and disinfecting the farm environment after the outbreak could effectively reduce the virus load in the farm environment.

Almost positive samples were found in primiparous sow pens. These results could explain the gilt management quality that those gilts might not have enough PED immunity. Proper gilt management could be done by vaccination or feedback protocol program. However, it must be done with caution on reducing virus contamination in the farm environment.

Oral fluid sample is convenient when sampling. It would work well with pathogens found in saliva or respiratory pathogens. In case of PEDV, it would not work well if it had low feces contamination (Pepin et al., 2015). In this study, we did not have any positive samples from oral fluid samples since PEDV did not shed via saliva. In addition, individual sampling of oral fluid from those gilts and sows yielded

negative results. Fecal sampling would need only the plastic bags and could be collected directly from the rectum of individual pigs. Surface swab samples are very useful for contaminated fomites and could be used indirectly for piglet feces contamination on the pen floor when having diarrhea.

Performance indices of 3 collection times demonstrated the negative impact of PEDV towards the breeding and farrowing problems both at 1MAO and 2MAO. Lower farrowing rate with higher return rate and abortion rate showed negative impact in breeding herd because PED might cause pregnancy failure and fetal loss from suboptimal nutrient supply (Olanratmanee et al., 2010). Percentage of stillbirth per litter as a result of the pregnant females infected with PEDV during 91–120 days of pregnancy did affect only at 1MAO. Pre-weaning mortality rate could be due to low immunity piglet from low immunity late term pregnant sows when the gut feedback were done together with the remaining virus in the environment. The sow herd production parameters should recover to the baseline production in 10 weeks after the herd closure (Goede and Morrison, 2016).

In this study, external factors including PRRSV outbreak, flooding seasons, and worker problems could influence the PED management strategy of the studied farm and could affect the performance index parameters. However, from the onset of PED outbreak, the correct and rapid diagnosis along with the gut feedback protocol to raise the herd immunity homogeneously must be done within 1 week and the strict biosecurity preventing the introduction of the new virus and reducing the viral load in the affected farms must be done. The production parameters should be back to the normal baseline within a few months if done properly.

CHAPTER VI

CONCLUSION AND SUGGESTIONS

Based on the findings of this study, fecal samples and surface swab samples could be used for PEDV monitoring in the affected farm after the outbreak when using the feedback protocol. The highest prevalence of PEDV remaining was found in the farrowing barns and the pregnant sows. These results lead to the management at the right point for prevention of the re-outbreak. These selected 3 sample types showed pros and cons of the measurement. Oral fluid sampling method might not work well if the pigs have low feces contamination when collected individually. Fecal samples are the samples of choice since the virus shedding is through feces and pooled samples might increase the sensitivity of the findings. Additionally, surface swab samples are very useful to demonstrate the existence of the virus on the contaminated fomites and environment. It should be noted that there are many systems of the swine farms such as one site, two sites or three sites. When sampling, the farm veterinarians must evaluate the results if the objective of those collected samples could answer the farm problems. Again, sample collection must be conducted from low to high risk arears, respectively.

Further studies are suggested and could be as follows:

1. The information from this study reported only the viral residues in the studied swine farm. The serological test could make more clear picture for the monitoring. Unfortunately, the serological tests are not commercially available.

2. This study reports the qualitative results of the virus. For more information, qRT-PCR test could provide additional data for quantitative results.

3. The sampling types used in this study could apply for others disease monitoring if applicable.

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APPENDICES

Appendix A

RT-PCR assay and chemicals

1. RNA extraction

- VB Lysis buffer	400 µl
- Ad buffer	450 µl
- W1 buffer	400 µl
- Wash buffer	600 µl
- RNA-free water	50 µl

2. RT-PCR assay

- SuperScript™ III RT/Platinum™ Taq Mix
  - 2X Reaction Mix a buffer 50 µl
  - dNTPs (dATP, dCTP, dTTP, dGTP) 0.04mM of each
  - $MgSO_4$ 5mM
- Agarose gel (Vivantis®) Malaysia
  - Agarose (ultra-pure) 1.2/ 0.8g
  - 1x TAE buffer
- 50x TAE buffer
  - Tris-base 242.0g
  - Glacual acetic acid 57.1g
  - 0.5M EDTA (pH 8.0) 100.0ml
  - Distilled water 1000.0ml

3. Other chemicals

- TE buffer
- Tris (10 mM)
- EDTA (1 mM)
- Sodium Dodecyl Sulfate (Vivantis®) Malaysia

Appendix B

Raw data of performance index

Performance index	Before outbreak (Jan 2017)	During outbreak (July 2017)	After Outbreak (Jan 2018)
Breeding performance			
Total number of services	218	165	156
Percent repeat services	11.9	21.2	30.1
Percent multiple mating	100	100	100
Weaning-1st service interval	9.4	19.9	9.2
Percent sows bred by 7 days	87.3	7.2	89.1
Entry-1st service interval	153.2	110.8	59.5
Farrowing performance			
Number of sows farrowed	198	188	108
Average parity of farrowed sows	2.9	3.7	4.7
Average total pigs per litter	12.6	12.6	11.1
Average pigs born alive/litter	10.8	9.8	9.7
Average birth weight/liveborn pig	1.7	1.7	1.7
Percent still born pigs	11.4	19.2	11.6

Performance index	Before outbreak (Jan 2017)	During outbreak (July 2017)	After Outbreak (Jan 2018)
Percent mummies	2.8	3.1	0.9
Farrowing rate	80.5	75.2	51.4
Adjusted farrowing rate	82.2	77	52.9
Farrowing interval	150	152	155
Litter/mated female/year	2.25	1.91	2.35
Weaning performance			
Number of litters weaned	201	192	133
Total pigs weaned	2006	0	1212
Pigs weaned per sow	10	0	9.1
Pre-weaning mortality	4.6	100	8.5
Average piglet weaning weight	7.0	.	6.9
Average age at weaning	20.9	0	21.2
Adjusted 21 days litter weight	71	.	65
Pigs weaned/mated female/year	22.4	0	21.4
Pigs weaned/lifetime female	15	.	22

Performance index	Before outbreak (Jan 2017)	During outbreak (July 2017)	After outbreak (Jan 2018)
Population			
Ending female inventory	1158	970	886
Average parity	1.9	2.8	3.5
Average female inventory	1175.8	975.3	900.7
Average gilt pool inventory	163.1	20.4	26.2
Gilts entered (pigs)	18	0	0
Sows and gilts culled (pigs)	14	0	38
Sows and gilts deaths (pigs)	13	9	3
Ending boar inventory (pigs)	19	19	19
Sow-boar ratio	60.9	51.1	46.6
Replacement rate (%)	18.0	0	0
Culling rate	14.0	0	49.7
Death rate	13.0	10.9	3.9
Average non-production sow days	100.8	104.3	64.8
Average NPD/parity record	46.6	46.0	45

Appendix C

Farm Information

1. Production operation : 700-Sow herd / batch farrowing system
2. Closed barn with evaporative cooling system
3. Biosecurity assessment

Animal risk factors	not self-replacement
Feed and water risk factors	no risk
Owner family employees	no personal disinfection
Visitor risk factors	no personal disinfection
Site risk factors	high density farm area

4. Vaccination program

Gilt	PRRS (modified live)	3 days after entering into the farm
	PCV2	1 wk
	PRRS (live)	4 wk
	CSF+FMD	8 wk
Sows	CSF	2 wk after farrowing
	FMD	3 wk

5. Health statue before PED outbreak (June 2017)

AD Elisa test	Negative
CSF-NPLA test	GMT 9.6
PRRSV isolation	Negative
Semen quality	poor (bacterial contamination)
Water quality	Good (standard bacterial contamination level)

6. Barn disinfection protocol

Remove all equipments

High pressure water with detergent

High pressure water

Glutaraldehyde 1:100

Downtime 5-7 days

7. History of the PED outbreak

On June 26th 2017 veterinarian was notified from farm that have the watery diarrhea in all piglets of 3 sows in 1 farrowed barn. Farm checked the piglets feces by PED commercial kits and the results show all positive and then they sent the clinical sign piglets to laboratory for confirm by RT-PCR assay. Laboratory results released in next day as positive PED and in this day they prepared equipments for gut feedback protocol in the next day.

On June 28th 2017 the clinical sign piglets found in every farrowed barn and the feedback protocol started from this day for 5 consecutive days after finished feedback protocol farm start intensive disinfection all over the farm by limited people to enter the farm, sprayed vehicles and equipment before entering farm, cleaning and spray disinfection in all barn once a day along with culling infected piglets.

After done the feedback protocol about 20% of sows show diarrhea and recovery within 1 week but the clinical sign in piglets show as sporadic until 4 months after outbreak. Then the farm had flood problem and worker problem followed by PRRS outbreak at the end of the year.

8. Gut feedback protocol

   i. Calculate the amount of feedback required by

   ii. Total sows in farm (700) x amount feedback/day (10ml) = 7000 ml/7L

   iii. Use 1 intestine : 1 dechlorinated water for total 7L

   iv. So use 3.5 kg of intestine from infected piglets mixed with 3.5 L of dechlorinated water

   v. Mince mixture together and added antibiotic for kill bacteria

   vi. Feed the mixture to all sows in farm 10 ml each

   vii. Repeat this protocol and feeding to all sows for 5 consecutive day

VITA

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