Final report

Project Title Interference of replication of porcine reproductive and respiratory distress virus (PRRSV) by swine scFv specific to the viral nonstructural protein-1

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Final Report

Project Title Interference of replication of porcine reproductive and respiratory distress virus (PRRSV) by swine scFv specific to the viral nonstructural protein-1

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Abstract

Project Code:

Project Title: Interference of replication of porcine reproductive and respiratory distress virus (PRRSV) by swine scFv specific to the viral nonstructural protein-1

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Project Period: June 2013-June 2015

Abstract:

Porcine reproductive and respiratory distress syndrome virus (PRRSV) infects swine and causes highly infectious disease, *i.e*., porcine reproductive and respiratory distress syndrome (PRRS), which has enormous economic impact on the global swine industry. PRRSV infected sows causing reproductive failure while infected piglets and growing pigs are succumb severe respiratory problem. The infecting host has prolonged viremia and persistent infection indicating that the overall immune responses are incapable of completely eliminating the virus. Among 14 functionally different non-structural proteins (nsp), nsp1 is reported to be a multifunction protein containing 2 self-releasing proteins; nsp1α and nsp1β. In this study, a phage library displaying porcine single chain antibody (p-scFv) fragments was successfully constructed using immunoglobulin genes of multiple pigs. Recombinant nsp1β protein of PRRSV was produced and used as a panning antigen for selecting phage clones that display nsp1β specific-p-scFv from the phage display library. Gene sequence coding for the nsp1β specific-p-scFv was molecularly linked to a DNA sequence coding for a cell penetrating peptide (CPP); Penetratin (PEN). The PEN-p-scFv was tested for their ability to hamper the PRRSV replication in infected MARC-145 cell. The infected MARC-145 cells treated with PEN-p-scFv no.5 has significantly lowest amount of infected foci when compare to control PEN-p-scFv, neutralizing pig serum, Poly:IC. The obtained PEN-p-scFv has potential activity for interfering with PRRSV replication. However, the molecular of inhibition should be further study in order to understand for treatment remedy discovery.

Keywords : PRRSV, nsp1, phage display scFv, cell penetrating peptide

Executive summary

Porcine reproductive and respiratory distress syndrome virus (PRRSV) is a causative agent of porcine reproductive and respiratory distress syndrome (PRRS), characterized by severe reproductive failure in breeding stocks, *i.e*., infertility, late-term abortion, mummified fetus, stillbirth, and/or the birth of weak newborns that often died from the respiratory distress. The virus can induce prolonged viremia and persistent infection. The disease is recognized as the most economically important infectious disease of pigs worldwide including Thailand. Currently, PRRSV isolates are divided into two distinct genotypes; European (EU) or type I and the North America (NA) or type II. In Thailand, both genotypes have been reported. At present, there is no specific treatment of PRRS. Available PRRS vaccines (live attenuated and inactivated vaccines) confer limited protective efficacy. Among 14 different functionally functions, non-structural protein 1 (nsp1) is the first viral protein synthesized during PRRSV replication. The nsp1 can be auto-cleaved into nsp1α and nsp1β by papain-like cysteine proteases (PCPα and PCPβ), respectively. The nsp1α is 180 amino acid long and has two zinc-finger (ZF) domains, the cysteine protease (PCPα) domain and a C-terminal extension (CTE). The nsp1β has 202 amino acids in length and has three known domains: 1) N-terminal nuclease domain (NTD), 2) middle linker domain and 3) C-terminal PCPβ domain. Previous study has been shown that the correct processing of the nsp1α-nsp1β and nsp1β-nsp2 cleavage sites is essential for PRRSV genome replication. In human, antibody therapy has been practiced in medicine for more than a century. However, in field of swine production; antibody therapy still not in attention.

This study try to construct porcine scFv (p-scFv) phage display library for use as a biological tool for in the *in vitro* production of porcine antibodies of desired specificities. Production of nsp1 specific-p-scFv that inhibit the PRRSV replication with the ultimate aim of developing further as a sole or adjunctive remedy for treatment of PRRS. This research is focus to produce cell penetrating single chain variable fragment antibody of porcine (PEN-p-scFv) specific to non-structural protein 1 β (nsp1β) of PRRSV in order to hamper viral replication. Predicted and/or cloned porcine immunoglobulin sequences were retrieved from GENBANK database for *vh/vl* oligonucleotide primer sequences design. Total RNA was extracted from porcine PBMC, synthesized to cDNA, *vh/vl* amplification and construction of porcine-*scfv (p-scfv*) phage display library.

Thai highly pathogenic PRRSV; designated HP/Thailand/19500LL/2010 (TH19500LL/10) was propagated in MARC-145 cells. Total RNA was extracted and synthesized to cDNA. The nps1β was amplified and introduced into E. coli. Recombinant nps1β protein was induced to overexpress and purified. The purified recombinant nps1β protein was used to select specific p-scFv from library. The

cell penetrable specific p-scFv or PEN-p-scFv was produced and test for their efficacy in interfere PRRSV replication.

The results show that the infected MARC-145 cells treated with PEN-p-scFv no.5 has significantly lowest amount of infected foci when compare to control PEN-p-scFv, neutralizing pig serum, Poly:IC. The obtained PEN-p-scFv has potential activity for interfering with PRRSV replication. However, the molecular of inhibition should be further study in order to understand for treatment remedy discovery.

Objective

Aims

1. Construction of porcine scFv (p-scFv) phage display library for use as a biological tool for the *in vitro* production of porcine antibodies of desired specificities
2. Production of nsp1 specific-p-scFv that inhibit the PRRSV replication with the ultimate aim of developing further as a sole or adjunctive remedy for treatment of PRRS

Specific objectives

1. To construct p-scFv phage display library
2. To produce recombinant nsp1 of the PRRSV
3. To select phage clones that carry nsp1 specific-p-scFv from the so-constructed p-scFv phage display library
4. To produce cell penetrable p-scFv specific to nsp1 of PRRSV, i.e., PEN-p-scFv
5. To test the efficacy of the nsp1 specific-PEN-p-scFv in interfering the PRRSV replication

Research methodology

Methodology of specific objective 1: To construct porcine scFv (p-scFv) phage display library

1.1 Bioinformatics and sequence alignment for porcine immunoglobulin and oligonucleotide primers design

Predicted and/or cloned porcine immunoglobulin sequences were retrieved from GENBANK database. Nucleotide sequences alignment were performed using the web-based multiple sequence alignment program Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). VH primers were designed to anneal at Framework 1 (FR1) sequence and J_H segment. VL_K primers were designed to anneal at FR1 and FR4. Restriction endonuclease enzyme *Sfi*I and *Not*I restriction sites were flanked at 5' end of individual forward and reverse primers, respectively.

1.2 Amplification of porcine *vh/vl* sequences and construction of porcine *scfv* (*pscfv*) repertoire

Five ml of blood samples were collected individually from 6 pigs (aged 7 weeks old). These pigs had been vaccinated with circovirus vaccine at 2 weeks old, mycoplasma + classical swine fever vaccine at 3 weeks old and classical swine fever vaccine at 6 weeks old. Peripheral blood mononuclear cells (PBMCs) were collected by using Ficoll-Paque (GE Healthcare).

Total RNA was extracted from PBMCs of individual pigs by using Trizol® reagent and reverse transcribed to cDNA by using SuperScriptIII® (Invitrogen) according to manufacturer instruction. PCR amplification of porcine *vh* and *vl* sequences were performed using pairs of forward and reverse degenerate primers under appropriate condition.

The PCR reaction mixture (25 µl) consisted of:

Reagents	Volume (µl)	Final concentration
10x *Taq* polymerase buffer	2.5	1x
25 mM $MgCl_2$	1.5	1.5 mM
2 mM each dNTP	2	0.1 mM
10 µM Forward primer	1	0.2 µM
10 µM Reverse primer	1	0.2 µM
Taq polymerase (10 U/µl)	0.5	5 units
cDNA template	1	-
dH_2O	15.5	-
Total	25	

PCR condition for *vh* and *vl* amplification was: initial denaturation at 94ºC for 5 minutes, followed by 30 cycles of denaturation at 94ºC for 1 minute, annealing at 50ºC for 1 minute, and extension at 72ºC for 1 minute. The final extension was performed at 72ºC for 10 minutes.

The amplified products of *vh* and *vl* were verified by agarose gel electrophoresis and ethidium bromide staining. The expected size of *vh* and *vl* were excised from agarose gel slabs and purified by using Agarose Gel Extraction kit DNA cleanup (Jena Bioscience, Germany). All purified products were pooled and used as the template for p-*scfv* production by spliced overlapped extension PCR (SOE-PCR). Repertoire of the p-*scfv* was produced by linking randomly the *vh* and *vl* sequences *via* a polynucleotide linker coding for $(Gly4Ser1)_3$ using PCR.

The PCR reaction mixture consisted of:

Reagents	Volume (µl)	Final concentration
10x *Taq* polymerase buffer	2.5	1x
25 mM $MgCl_2$	1.5	1.5 mM
2 mM each dNTP	2	0.1 mM
10 µM Forward primer	1	0.2 µM
10 µM Reverse primer	1	0.2 µM
Taq polymerase (10 U/µl)	0.5	5 units
Pooled *vh* template	0.5	2.5 ng
Pooled *vl* template	0.5	2.5 ng
dH_2O	15.5	-
Total	25	

PCR condition for linking the *vh* and *vl* sequences was: initial denaturation at 94ºC for 5 minutes, followed by 10 cycles of denaturation at 94ºC for 1 minute, annealing at 55ºC for 1 minute, and extension at 72ºC for 1 minute. Forward primer of *vh* and reverse primer of *vl* were added at this step and followed by 20 cycles of denaturation at 94ºC for 1 minute, annealing at 55ºC for 1 minute, and extension at 72ºC for 1 minute The final extension was performed at 72ºC for 10 minutes. The amplified products were verified by agarose gel electrophoresis.

1.3 Introduction of the p-scfv into suppressor *E. coli*, phage rescue and construction of p-scFv phage display library

The p-*scfv* amplicons were extracted from the agarose gel slabs and purified. The purified DNA was digested with *Sfi*I and *Not*I endonucleases and ligated into a phagemid vector pre-cut with the same enzymes. The ligation mixture was kept at 16 ºC for 18 hours and then was purified by using PCR purification kit (Jena Bioscience) according to manufacturer instruction. The purified product was introduced into competent TG1 *E. coli*. The p-scFv-displaying phage particles were rescued by co-infecting the phagemid transformed *E. coli* with a helper phage. The transformed *E. coli* was randomly selected and the presence of p-*scfv* was determined by using R1 and R2 primers

which have the annealing sites within the vector sequence. The expected size of the p-*scfv* by using this pair of primers was about 1000 bp.

Methodology of specific objective 2: To produce recombinant nsp1 of the PRRSV

2.1 Propagation of PRRSV

Thai highly pathogenic PRRSV; designated HP/Thailand/19500LL/2010 (TH19500LL/10) stock was provided by Prof. Dr. Porntippa Lekcharoensuk, the Department of Microbiology and Immunology, Faculty of Veterinary Medicine, Kasetsart University, Bangkok, Thailand. The virus was propagated in MARC-145 cells. The supernatant contains PRRSV was collected and kept at -80°C until use. The titer of virus was determined as reciprocal of Median Tissue Culture Infective Dose ($TCID_{50}$) per ml using Reed and Muench method.

2.2 Amplification of nsp1-coding sequences

Total RNA was extracted from the PRRSV and reverse-transcribed to cDNA. The nsp1 has two auto-cleavage sites to produce nsp1α- and nsp1β proteins. From data, nsp1β was reported to be a determining factor in the virulence of PRRSV, plays important role in PRRSV pathogenesis by subversion of host immunity. Thus, specific oligonucleotide primers for amplification of the *nsp1β* were designed from DNA sequence coding for the PRRSV nsp1 of the GENBANK database. *EcoR*I and *Xho*I endonuclease restriction recognition sites were incorporated into 5’ ends of the forward and the reverse primers, respectively for DNA cloning purpose. The nsp1β-coding sequences were amplified by PCR using the *nsp1β*-specific primers and the cDNA as the template.

The PCR reaction mixtures consisted of:

Reagents	Volume (µl)	Final concentration
10x *Taq* polymerase buffer	2.5	1x
25 mM $MgCl_2$	1.5	1.5 mM
2 mM each dNTP	2	0.1 mM
10 µM Forward primer	1	0.2 µM
10 µM Reverse primer	1	0.2 µM
Taq polymerase (10 U/µl)	0.5	5 units
cDNA template	1	-
dH_2O	15.5	-
Total	25	

PCR condition for the *nsp1β* amplification was: initial denaturation at 94ºC for 5 minutes, followed by 30 cycles of denaturation at 94ºC for 1 minute, annealing at 50ºC for 1 minute, and extension at 72ºC for 1 minute. The final extension was performed at 72ºC for 10 minutes. The amplified products were verified by agarose gel electrophoresis and ethidium bromide staining.

2.3 Cloning of nsp1β-coding sequences into bacterial vector

The PCR amplified product from **Section 2.2** was purified from the gel pieces containing the DNA bands before digesting with the respective restriction endonucleases. The digested products were ligated individually to pGEX-5X-3 expression vectors (GE Life Sciences) pre-digested with the same restriction endonucleases. Thereafter, the recombinant vectors were introduced into competent protein expression DH5α *E. coli* host. In order to verify the *E. coli* clones that harboring DNA inserts, PCR was performed by using the same condition as **Section 2.2**.

2.4 The nsp1-β recombinant protein expression

Small scale expression culture

A single colony of *E. coli* transformant contains *nsp1-β* recombinant vectors were individually inoculated into 5 ml of LB broth containing 100 µg/ml ampicillin and incubated at 28°C with shaking at 250 rpm for 18 hours. One hundred microliters of the overnight grown *E. coli* culture of each

clone was inoculated into 10 ml of LB broth containing 100 µg/ml ampicillin. The culture was incubated at 28°C with shaking at 250 rpm until the A_{600} reached 0.3-0.4 and then IPTG was added to a final concentration of 0.5 mM. The culture was further incubated at 28°C with shaking at 250 rpm for 3 hours. The bacterial cells were harvested by centrifugation. The cell pellet was suspended in 500 µl of PBS and lysed by sonication. The soluble part (supernatant) was collected and subjected to the protein analysis by SDS-PAGE and Western blotting. The expected size of nsp1-β recombinant protein was ~ 23 kDa. However, when expressed as GST-fusion protein (GST size ~ 25 kDa), the expected size of nsp1-β recombinant protein was ~ 48 kDa.

Large scale expression culture

From small scale expression, *E. coli* colony no. 1 that carried the *nsp1-β* recombinant vector was inoculated into 10 ml of LB broth containing 100 µg/ml ampicillin and incubated for 18 hours at 28°C with shaking at 250 rpm. Two milliliters of the *E. coli* culture was inoculated into 200 ml of LB broth containing 100 µg/ml ampicillin and incubated at 28°C with shaking at 250 rpm until the A_{600} nm reached 0.3-0.4. IPTG was added to a final concentration of 0.5 mM. The *E. coli* culture was further incubated at 28°C with shaking at 250 rpm for another 3 hours. The bacterial cells were then harvested.

2.5 The nsp1-β recombinant protein purification

The cell pellets (~ 1 g wet weight) from **Section 2.4** was suspended in 10 ml of native lysis buffer. The cells were lysed by sonication. The bacterial lysate was collected and verified by SDS-PAGE and Western blot analysis. The remaining lysate was loaded into affinity column previously washed with distilled water (DW) and equilibrated in the native lysis buffer. The column was washed with 5 volumes of the wash buffer. The nsp1-β recombinant protein was batchly eluted with 5 volumes of elusion buffer. The eluates were collected in 1 ml fractions. The fractions containing the nsp1-β recombinant protein were detected by SDS-PAGE and Coomassie Brilliant blue G-250 (CBB-G250) staining and Western blotting probed with anti-GST tag.

Methodology of specific objective 3: To select p-scFv specific to nsp1-β recombinant proteins

3.1 Phage bio-panning

Five microgram of purified Nsp1-β recombinant protein was immobilized onto ELISA plate wells by using coating buffer. The *E. coli* lysate contained recombinant vector (without *p-scfv* gene insert) was used as subtractive component. The unbound phages to *E. coli* lysate carrying empty vector were collected and allowed to bind to purified nsp1-β recombinant protein in the coated

ELISA wells. After extensive washing away the unbound phages with PBST, the log phase grown HB2151 *E. coli* cells were added into the well and phage transduction was allowed for 20 minutes. The preparations were collected and spread onto 2x YT-AG plates and the plates were incubated at 37^{O}C overnight.

3.2 Screening of HB2151 *E. coli* transformants harboring recombinant *p-scfv*-phagemids

Well isolated transformed HB2151 *E. coli* colonies on the selective plates of **Section 3.1** were randomly selected and used as template for PCR amplification of the *p-scfv* inserts in the recombinant phagemids contained in individual *E. coli* colonies. The oligonucleotide primers used in the PCR for *p-scfv* amplification were: forward (*R1*) and reverse (*R2*) primers which were the primer sequences contained in the phagemid vector. The selected HB2151 *E. coli* colonies (positive for *p-scfv* amplicons at ~1,000 bp) were streaked onto a 2x YT-AG replica plate and grown at 37^{O}C overnight. The bacteria were subsequently tested for their ability to produce p-scFv protein.

3.3 Screening of the *p-scfv*-phagemid *E. coli* transformants that can express p-scFv

The bacterial colonies harboring recombinant *p-scfv*-phagemids were individually grown in 2x YT-A broth incubated at 37^{O}C until they become log phase. Over expression of the p-scFv protein was induced by using IPTG 0.2 mM final concentration. The bacterial cells were further incubated at 37^{O}C for 3 h before collecting of the bacterial cell pellet. The bacterial cells were broken by using ultrasonicator. The soluble fraction revealed p-scFv expression by Western blotting using anti-E tag antibody. The expected molecular weight of the p-scFv was about 25-30 kDa.

3.4 Screening of p-scFv that bound specifically to nsp1-β

The *E. coli* lysates containing soluble p-scFv was screened for the clones that their expressed p-scFv can bind to recombinant nsp1-β antigens by indirect ELISA. The *E. coli* clones containing p-scFv protein that specifically bound to recombinant nsp1-β proteins were sub-cloned to produce cell penetrable p-scFv (PEN-p-scFv).

Methodology of specific objective 4: Sub-cloning of the p-*scfv* from the phagemid to a plasmid carrying DNA coding for cell penetrating peptide (CPP)

4.1 Subcloning of the *pscfv* from the phagemid to a plasmid carrying DNA coding for cell penetrating peptide (CPP)

From data had shown that nsp1 protein was expressed in infected cells. In order to produce cell penetrable p-scFv, the p-*scfv* in the p-*scfv*-phagemid transformed *E. coli* from **Section 3.4** was

sub-cloned into a bacterial expression vector backbone established in the Mentor's laboratory. This plasmid, *pen*-pET23b+, carries a DNA sequence coding for a 16 amino acid peptide called Penetratin (PEN) which is a cell penetrating hydrophobic peptide. The recombinant phagemid was extracted from *E. coli* of **Section 3.4** and digested with appropriate restriction endonucleases before ligating with the *pen*-pET23b+ vector. The *pen-p-scfv*-pET23b+ vector were introduced into competent BL21(DE3) *E. coli* expression host. Transformed *E. coli* carrying the recombinant plasmid was selected by PCR**.**

4.2 Expression and purification of PEN-p-scFv

The selected *E. coli* transformants with *pen-p-scfv*-pET23b+vector were grown under IPTG induction and the cell penetrating-p-scFv (PEN-p-scFv) specific to nsp1-β was purified from their lysates by affinity resin (the recombinant PEN-p-scFv is 6x-His-tagged). The purified recombinant proteins were detected by using SDS-PAGE and Western blotting.

Methodology of specific objective 5: Determination of efficacy of the PEN-p-scFv specific to nsp1 (nsp1-α and/or nsp1-β) in PRRSV replication inhibition

5.1 Cells line and virus

The African green monkey kidney derived cells; MARC-145 was used in this study. The MARC-145 cells were maintained in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco) supplemented with 1% glutamine, 10% FBS, 1% Penicillin/Streptomycin (complete IMDM).

Thai highly pathogenic PRRSV; HP/Thailand/19500LL/2010 (TH19500LL/10) was used to infected MARC-145 cells for determination of neutralizing activity of PEN-p-scFv antibody against PRRSV infection.

Both MARC-145 cells and PRRSV; Thai isolated strain were kindly provided by Prof. Dr. Porntippa Lekcharoensuk, the Department of Microbiology and Immunology, Faculty of Veterinary Medicine, Kasetsart University, Bangkok, Thailand.

5.2 Neutralization test of PRRSV infected MARC-145 cells by PEN-p-scFv antibody

MARC-145 cells were seeded in 12 wells culture plate which were maintained in complete IMDM. A monolayer MARC-145 cells were washed with PBS. Five hundred microliter (5.8 $TCID_{50}$/ml) of the virus stock from **Section 2.1** was inoculated onto MARC-145 cells. The culture plates were culture at 37°C, 5% CO_2 incubator for 1 h. The infected MARC-145 cells were supplemented with complete IMDM containing 10 µg of purified PEN-p-scFv from individual *E. coli* clone and control PEN-p-scFv. The PRRSV infected-pig serum was use as positive inhibition control (neutralizing antibody titer 64). The MARC-145 cells added with medium alone was use as negative

control. The present of PRRSV in infected cells were determined by using immunoperoxidase monolayer assay (IPMA) at 3 days post infection.

5.3 Detection of PRRSV infected MARC-145 cells by using IPMA

After day 3 post infection, the present of PRRSV in infected cells were determined by using IPMA. Cells were washed with PBS and fixed with 20% cold methanol for 30 minutes. Cells were washed with PBS before adding 1%H_2O_2. Cells were kept in dark for 30 minutes and washed with PBS containing Tween-80 (PBS-T). PRRSV infected pig serum was used to detect PRRSV in infected MARC-145 cells. The color was developed by adding goat anti-pig Ig-HRP conjugated and DAB substrate solution (1:1,000). The plate was kept in dark for 30 minutes before stop reaction by H_2O. The cells were revealed under light microscopy.

Results

1. Construction of porcine scFv (p-scFv) phage display library

1.1 Bioinformatics and sequence alignment for porcine immunoglobulin and oligonucleotide primers design

Sequences of immunoglobulin were retrieved from GENBANK database. Immunoglobulin amino acid sequences alignment shown similar fashion as other species that highest variation sequences were found in Complementary Determining Region (CDR) especially CDR3 as shown in **Figure 1**. VH primers were designed to anneal at Framework 1 (FR1) sequence and J_H segment. VL_K primers were designed to anneal at FR1 and FR4. Restriction endonuclease enzyme *Sfi*I and *Not*I restriction sites were flanked at 5' end of individual forward and reverse primers, respectively. The designed primer sequences were shown in **Table 1**.

1.2 Amplification of porcine *vh/vl* sequences and construction of porcine *scfv* (*pscfv*) repertoire

Porcine *vh/vl* sequences were amplified by using PCR. The amplified products of *vh* and *vl* were verified by agarose gel electrophoresis and ethidium bromide staining as shown in **Figures 2** and **3,** respectively. The *vh* and *vl* amplicons were extracted from the agarose gel slabs, purified by using Agarose Gel Extraction kit DNA cleanup (Jena Bioscience, Germany). All purified products were pooled and used as the template for *pscfv* production by spliced overlapped extension PCR (SOE-PCR). Repertoire of the *pscfv* was produced by linking randomly the *vh* and *vl* sequences *via* a polynucleotide linker coding for $(Gly4Ser1)_3$ using PCR as shown in **Figure 4**.

1.3 Introduction of the p-scfv into suppressor *E. coli*, phage rescue and construction of p-scFv phage display library

The *p-scfv* amplicons were extracted from the agarose gel slabs and purified. The purified DNA was digested with *Sfi*I and *Not*I endonucleases and ligated into a phagemid vector pre-cut with the same enzymes. The ligation mixture was kept at 16 ºC for 18 hours and then was purified by using PCR purification kit (Jena Bioscience) according to manufacturer instruction. The purified product was introduced into competent TG1 *E. coli*. The pScFv-displaying phage particles were rescued by co-infecting the phagemid transformed *E. coli* with a helper phage. The transformed *E. coli* was randomly selected and the presence of *pscfv* was determined by using R1 and R2 primers which have the annealing sites within the vector sequence. The expected size of the *pscfv* by using this pair of primers was about 1000 bp **(Figuer 5).** The titer of the rescued phage repertoire was 2.5 x 10^7 cfu/ml. The diversity of *pscfv* repertoire was determined by digestng the *pscfv* amplicon with *Mva*I endonuclease **(Figure 6).**

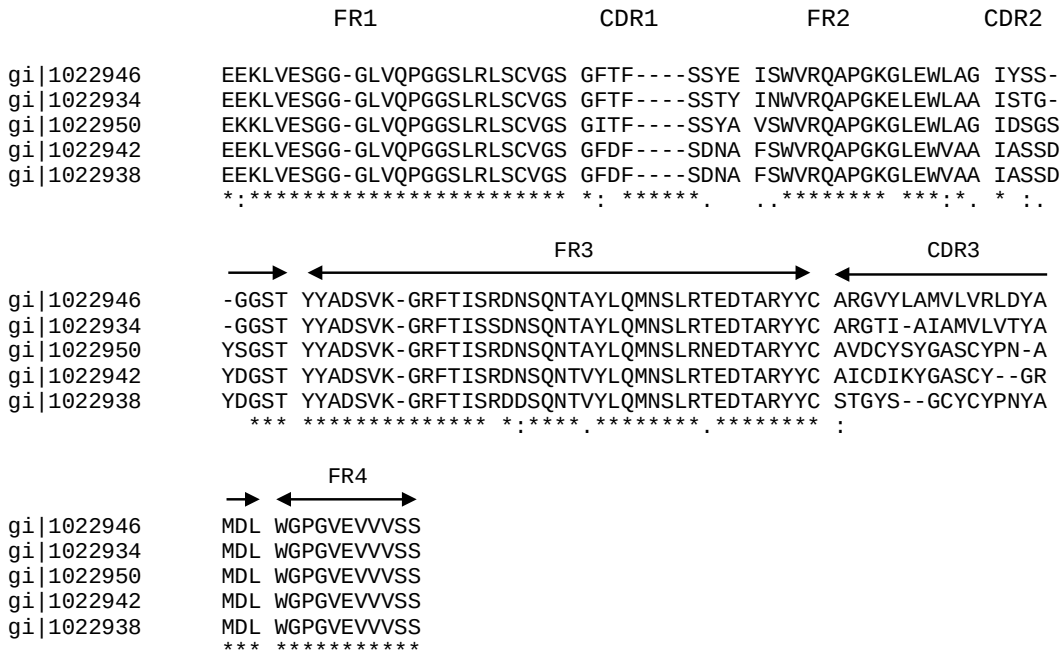

Figure 1 Example of multiple amino acid sequences alignment of swine immunoglobulin variable region

Table 1. Oligonucleotide primer sequences for porcine *vh* and *vl* amplification

Primer name	Sequence
VHF	5'-GGGCCCAGCCGGCCGAG**R**AGAAGCTGGTGGAGTC-3'
JHR	5'-AGATCCGCCGCCACCCGACCCACCACCGCCCGAGCCACCGCCACCT TGAGGACACGACGACTTCAACGCCTGG-3'
VLF1	5'-GGTGGCGGTGGCTCGGGCGGTGGTGGGTCGGGTGGCGGCGGATCT AGGTCCAGTCA**S**AGCCTTGAG-3'
VLF2	5'-GGTGGCGGTGGCTCGGGCGGTGGTGGGTCGGGTGGCGGCGGATCT AGGTCCAGT**M**AGAGCCTC**S**TAGAC-3'
VLR	5'-CCTGCGGCCGCTTTGA**KY**TCCAGATTGGTCCC-3'

Underlined alphabets of VHF and VLR are *Sfi*I and *Not*I restriction endonuclease sites, respectively

Bold alphabets are degenerate nucleotides

R, A or G; **S**, G or C; **M**, A or C; **K**, G or T and **Y**, C or T

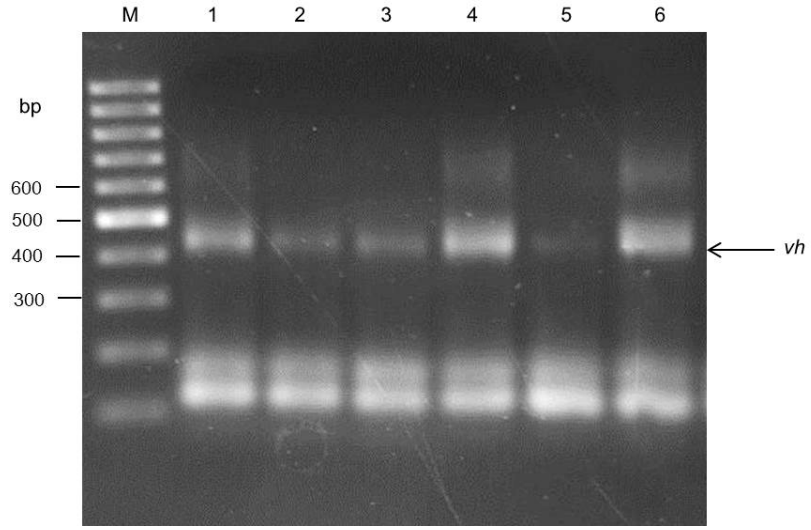

Figure 2 PCR products of VH-coding DNA sequences

Lane M, GeneRuler 100 bp DNA ladder

Lanes 1-6, *vh* PCR products from cDNA of pig nos. 1-6, respectively

The size of each PCR product was about ~450 bp (indicated by arrow)

Numbers at the left are DNA sizes in bp

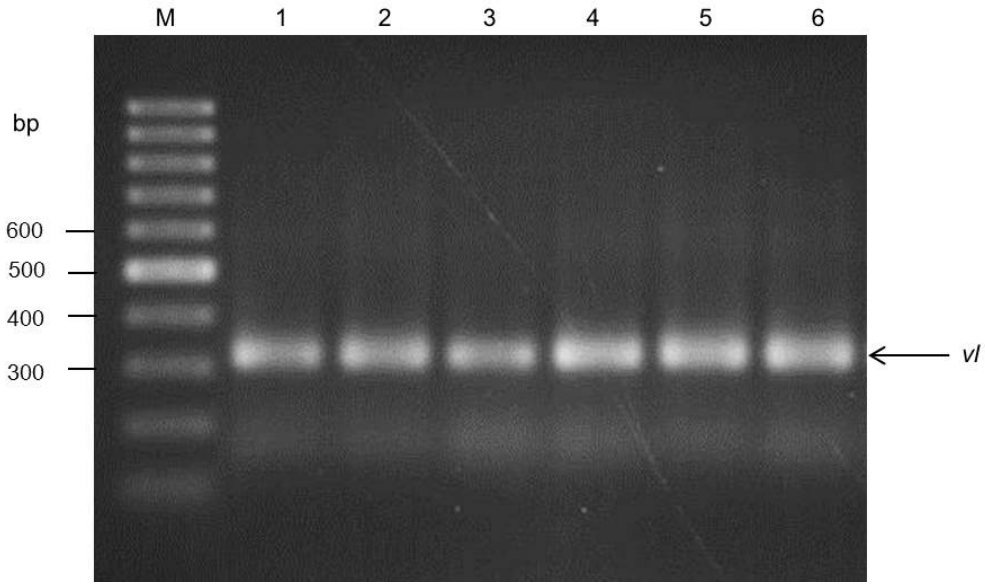

Figure 3 PCR products of VL-coding DNA sequences

Lane M, GeneRuler 100 bp DNA ladder

Lanes 1-6, *vl* PCR products from cDNA of pig nos. 1-6, respectively

The size of each PCR product was about ~350 bp (indicated by arrow)

Numbers at the left are DNA sizes in bp

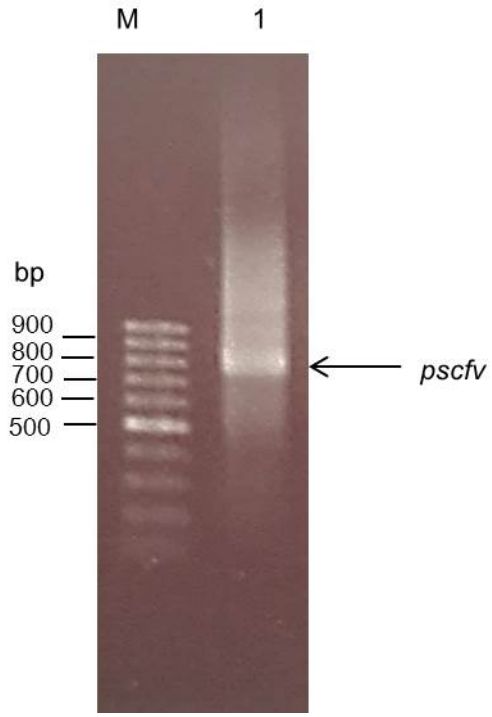

Figure 4 SOE-PCR products of p*scfv*-coding DNA sequences derived from randomly linked *vh* and *vl* sequences

Lane M, GeneRuler 100 bp DNA ladder

Lane 1, *pscfv* amplicon (~750 bp, arrow)

The size of each PCR product was about 750 bp

Numbers at the left are DNA sizes in bp

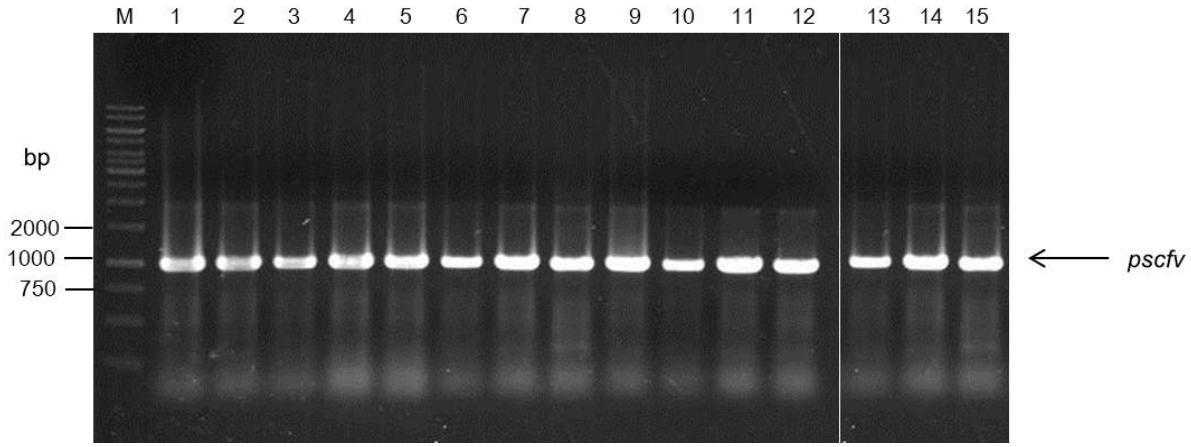

Figure 5 The *pscfv* amplicons derived from 15 *scfv*-phage transformed TG1 *E. coli* clones

Lane M, GeneRuler 1 kb DNA ladder

Lanes 1-15, *pscfv* amplicons from the transformed *E. coli* clones 1-15*,* respectively

The size of each PCR product was about ~1000 bp (indicated by arrow)

Numbers at the left are DNA sizes in bp

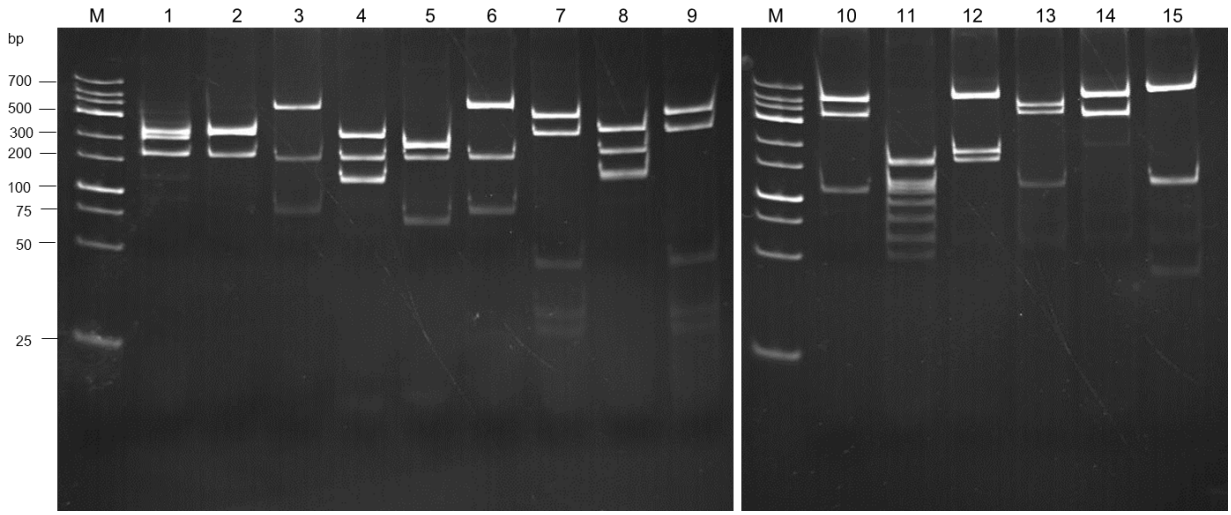

Figure 6 Examples of RFLP patterns of *pscfv* sequences from *scfv*-phage transformed TG1 *E. coli* clones

Lane M, GeneRuler^{TM} low range DNA ladder

Lanes 1-15, *Mva*I-cut-*pscfv* sequences which were amplified from individual transformed TG1 *E. coli* clone nos. 1-15, respectively.

Numbers of the left is DNA sizes in bp

Results

2. Production of recombinant nsp1 of the PRRSV

2.1 Propagation of PRRSV

Thai highly pathogenic PRRSV; HP/Thailand/19500LL/2010 (TH19500LL/10) was propagated in MARC-145 cells. The titer of virus was 5.8 $TCID_{50}$ per ml.

2.2 Amplification of nsp1-coding sequences

The Nsp1β-coding sequences wasamplified by PCR using the *nsp1β*-specific primers and the cDNA. The amplified products were verified by agarose gel electrophoresis and ethidium bromide staining. The expected size of nsp1β- amplicons was~ 550 bp, respectively **(Figure 7)**.

2.3 Cloning of nsp1β-coding sequence into bacterial vector

In order to verify the *E. coli* clones that harboring DNA inserts, PCR was performed by using the same condition as **M&M Section 2.2**. The amplified product was verified by agarose gel electrophoresis and ethidium bromide staining **(Figure 8)**. The *E. coli* clones that harboring DNA inserts at the expected sizes were selected for recombinant protein expression determination.

2.4 The nsp1-β recombinant protein expression

E. coli colony no. 1 from **Figure 8** that carried the *nsp1-β* recombinant vector was inoculated into LB broth containing 100 µg/ml ampicillin and incubated at 28°C with shaking at 250 rpm until the A_{600} nm reached 0.3-0.4. Over protein expression was induced by using IPTG to a final concentration of 0.5 mM. Result of SDS-PAGE and Western blot shown that the *E. coli* expressed nsp1-β recombinant protein in soluble fraction of *E. coli* lysate.

2.5 The nsp1-β recombinant protein purification

The bacterial lysate from **Section 2.4** was collected and verified by SDS-PAGE and Western blot analysis. The remaining lysate was loaded into affinity column previously washed with distilled water (DW) and equilibrated in the native lysis buffer. The column was washed with 5 volumes of the wash buffer. Recombinant nsp1-β protein was batchly eluted with 5 volumes of elusion buffer. The eluates were collected in 1 ml fractions. The fractions containing the recombinant nsp1-β protein were detected by SDS-PAGE and Coomassie Brilliant blue G-250 (CBB-G250) staining and Western blotting **(Figures 9** and **10).**

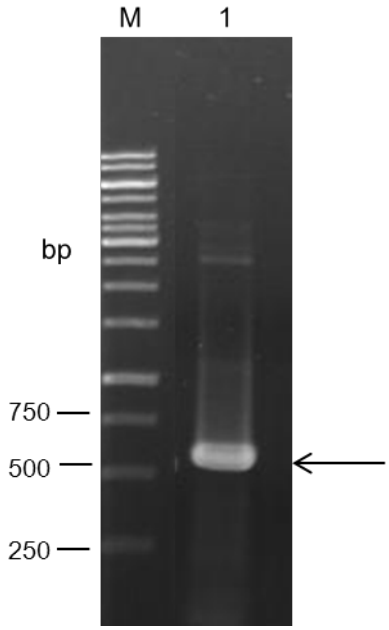

Figure 7 PCR products of nsp1β-coding DNA sequence

Lane M, GeneRuler 1 kp DNA ladder

Lane 1, PCR product of nsp1β-coding DNA sequences (~550 bp)

Numbers at the left are DNA sizes in bp

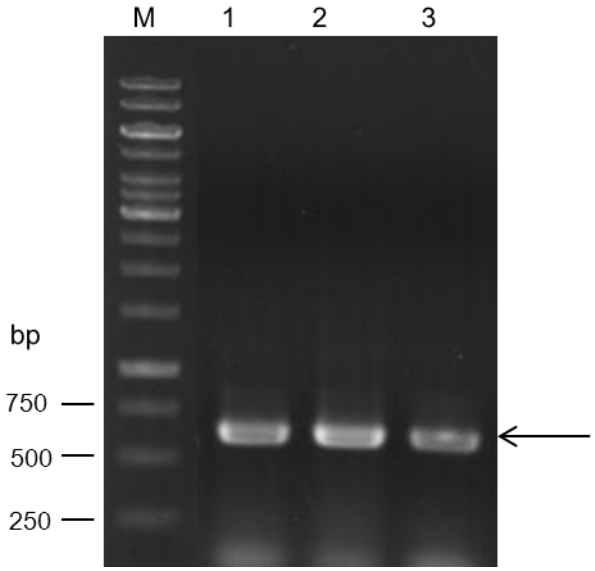

Figure 8 PCR products of nsp1β-coding DNA sequence for verification of the transformed *E. coli* clones that harbored DNA inserts

Lane M, GeneRuler 1 kp DNA ladder

Lanes 1-3, PCR products of nsp1β-coding DNA sequences from clone nos. 1-3, respectively

Numbers at the left are DNA sizes in bp

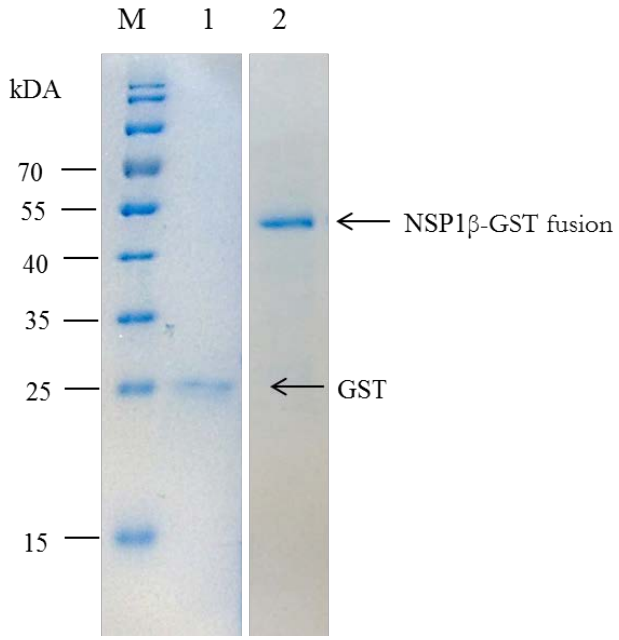

Figure 9 SDS-PAGE and Coomassie Brilliant blue G-250 staining of nsp1β-GST fusion recombinant protein for verification of purified nsp1β-GST fusion recombinant proteins

Lane M, Pre-stained protein marker

Lanes 1, SDS-PAGE and Coomassie Brilliant blue G-250 staining of purified GST recombinant protein (~ 25 kDa)

Lanes 2, SDS-PAGE and Coomassie Brilliant blue G-250 stained-purified nsp1β-GST fusion recombinant protein (~ 48 kDa)

Numbers at the left are protein sizes in kDa

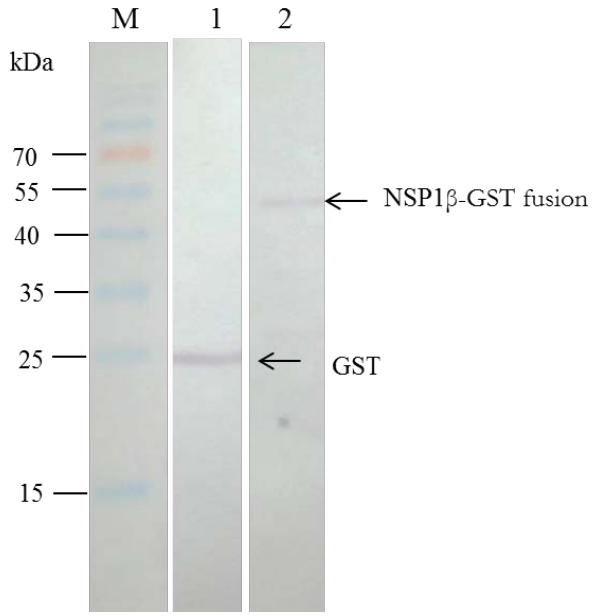

Figure 10 Western blotting of purified nsp1β-GST fusion recombinant protein

Lane M, Pre-stained protein marker

Lane 1, Western blot pattern of purified GST recombinant protein (~ 25 kDa)

Lane 2, Western blot pattern of purified nsp1β-GST fusion recombinant protein (~ 48 kDa)

Numbers at the left are protein sizes in kDa

Results

3. Selection of p-scFv specific to nsp1-β recombinant protein

After performed phage bio-panning, HB2151 *E. coli* transformants harboring recombinant *p-scfv*-phagemids were determined by using PCR and R1, R2 primers. By using nsp1-β recombinant protein as the antigen, from 12 selected *E. coli* clones 7 clones reveal p-*scfv* amplicons at ~ 1000 bp (50% positive) as shown in **Figure 11**. All 7 *E. coli* clones could express p-scFv recombinant protein at the expected molecular size (~25-30 kDa) as shown in **Figure 12**.

The *E. coli* lysates containing soluble p-scFv were screened for the clones that their expressed p-scFv can bind to recombinant nsp1-β antigen by indirect ELISA **(Figure 13)**. The *E. coli* clones containing p-scFv protein that specifically bound to recombinant nsp1-β and had complete *p-scfv* coding sequence was sub-cloned to produce cell penetrable p-scFv (PEN-p-scFv).

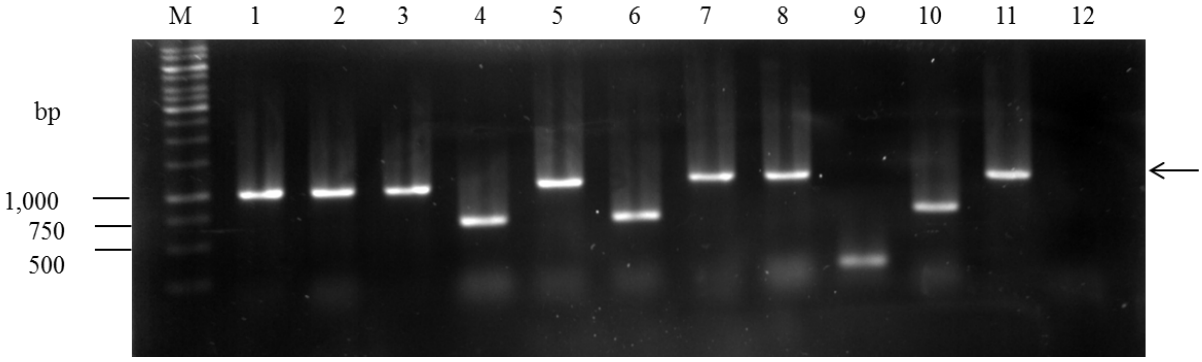

Figure 11 PCR products of p-scfv-coding DNA sequence for verification of the transformed *E. coli* clones that harbored recombinant *p-scfv*-phagemids

Lane M, GeneRuler 1 kp DNA ladder

Lanes 1-12, PCR products of p-scfv-coding DNA sequences from clone nos. 1-12, respectively (expected size ~ 1,000 bp)

Numbers at the left are DNA sizes in bp

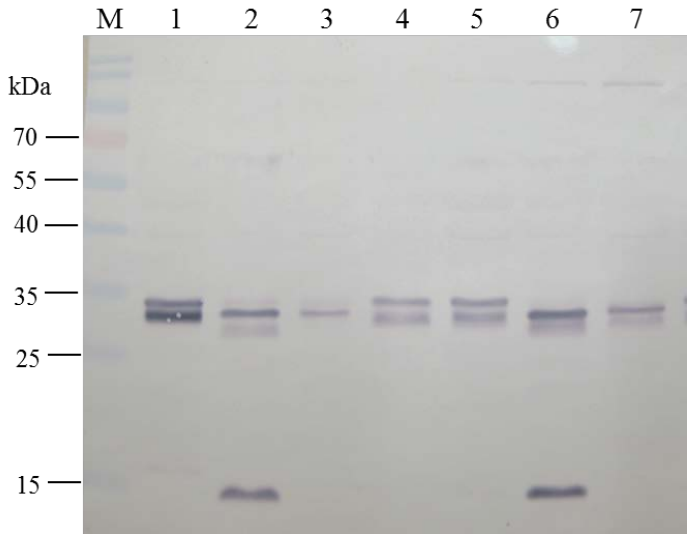

Figure 12 Western blotting pattern of expressed p-scFv recombinant protein

Lane M, Pre-stained protein marker

Lanes 1-12, Western blot pattern of expressed p-scFv recombinant protein from clone nos. 1-3, 5, 7-8 and 11, respectively (expected size ~ 25-30 kDa)

Numbers at the left are protein sizes in kDa

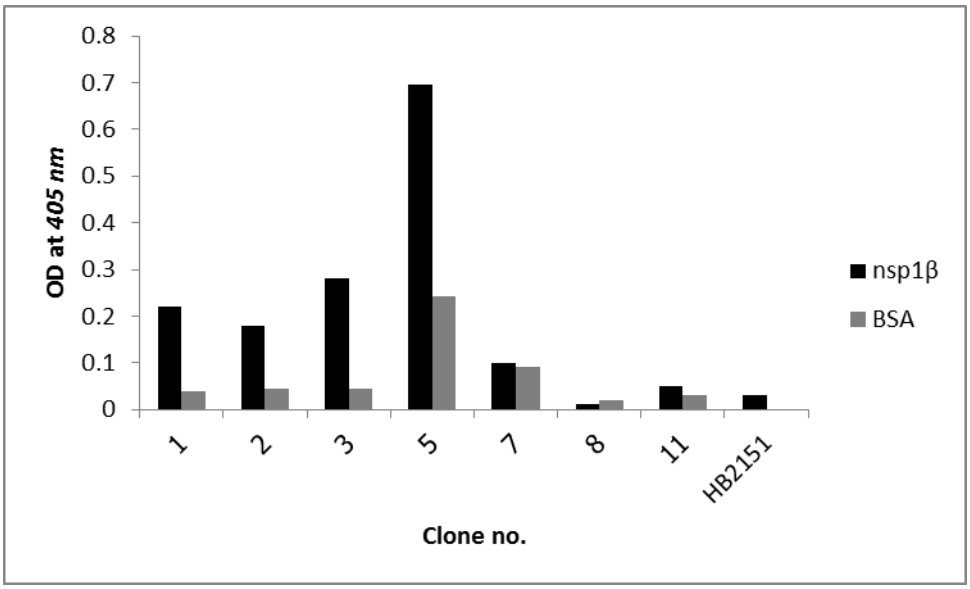

Figure 13 ELISA binding of p-scFv to nsp1-β recombinant protein.

BSA was used as irrelevant antigen control

HB2151 *E. coli* lysate was used as background (negative p-scFv) control

E. coli clones that gave OD $_{405nm}$ to nsp1-β recombinant protein more than two times higher than BSA were selected

4. Sub-cloning of the p-*scfv* from the phagemid to a plasmid carrying DNA coding for cell penetrating peptide (CPP)

4.1 Sub-cloning of the *p-scfv* from the phagemid to a plasmid carrying DNA coding for cell penetrating peptide (CPP)

Before sub-cloning of the *p-scfv* from the phagemid to a plasmid carrying DNA coding for cell penetrating peptide (CPP), DNA sequencing was determined. Only clone no.5 revealed complete *p-scfv* sequence as shown in **Figure 14**. The *p-scfv* sequence show 97% identity with V gene region of *Sus scrofa* IGHV1-15*01 F (accession no. AB513624). Thus in this study clone no.5 was selected for sub-cloning of the *p-scfv* from the phagemid to a plasmid carrying DNA coding for cell penetrating peptide (*pen-p-scfv*). Clone no.8 was selected as control p-scfv. The recombinant phagemid was extracted from *E. coli* of **Section 3.4** and digested with appropriate restriction endonucleases before ligating with the *pen*-pET23b+ vector. The *pen-pscfv*-pEt23b+ vector were introduced into competent *E. coli* expression host. Transformed *E. coli* carrying the recombinant plasmid was selected by PCR as shown in **Figure 15**.

4.2 Expression and purification of PEN-p-scFv

Clone no.1 of transformed *E. coli* carrying the recombinant plasmid was selected. The selected *E. coli* transformants with *pen-pscfv*-pEt23b+vector was grown under IPTG induction and the cell penetrating-p-scFv (PEN-p-scFv) specific to nsp1-β was purified from their lysates by affinity resin (the recombinant PEN-p-scFv is 6x-His-tagged). The purified recombinant nsp1-β protein was determined by Western blot as shown in **Figure 16**.

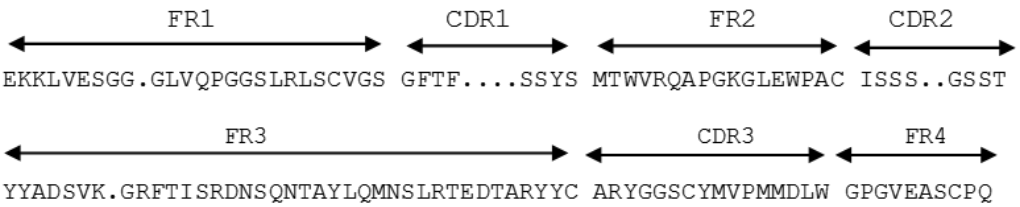

Figure 14 Deduced amino acid of *p-scfv* coding DNA sequence clone no.5

The *p-scfv* sequence show 97% identity with V gene region of *Sus scrofa* IGHV1-15*01 F (accession no. AB513624).

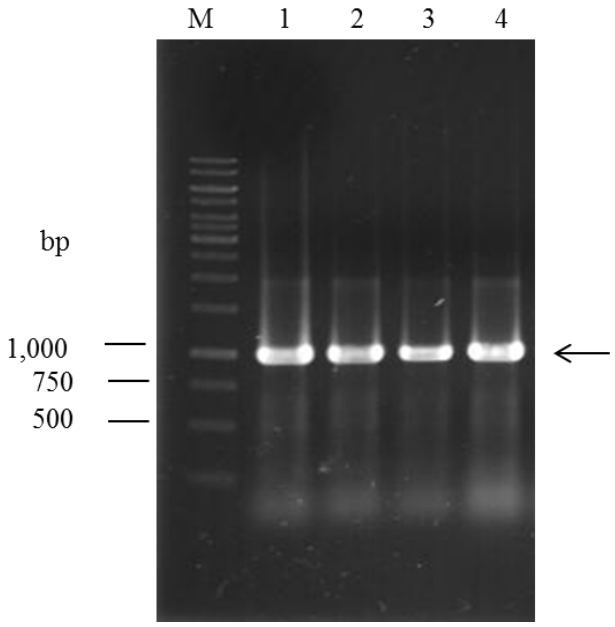

Figure 15 PCR products of screened transformed E. coli for verification of the transformed *E. coli* clones that harbored recombinant *pen-p-scfv* plasmid

Lane M, GeneRuler 1 kp DNA ladder

Lanes 1-4, PCR products of *pen-p-scfv*-coding DNA sequences from clone nos. 1-4, respectively (expected size ~ 1,000 bp)

Numbers at the left are DNA sizes in bp

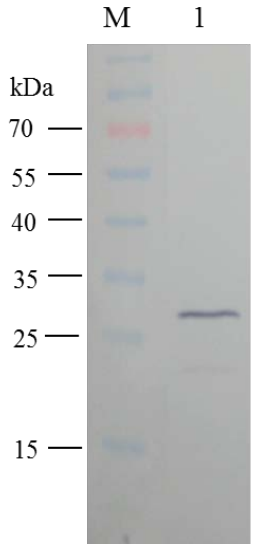

Figure 16 Western blotting of purified PEN-p-scFv recombinant protein

Lane M, Pre-stained protein marker

Lanes 1, Western blot of expressed purified PEN-p-scFv recombinant protein from clone no. 1-5 (expected size ~ 25-30 kDa)

Numbers at the left are protein sizes in kDa

5. Determination of efficacy of the PEN-p-scFv specific to nsp1-β in PRRSV replication inhibition

MARC-145 cells were seeded in 12 wells culture plate which were maintained in complete IMDM. A monolayer MARC-145 cells were washed with PBS. Five hundred microliter (5.8 $TCID_{50}$/ml) of the virus stock from **Section 2.1** was inoculated onto MARC-145 cells. The culture plates were culture at 37°C, 5% CO_2 incubator for 1 h. The infected MARC-145 cells were supplemented with complete IMDM containing 10 µg of purified PEN-p-scFv from individual *E. coli* clone and control PEN-p-scFv. The PRRSV infected-pig serum was use as positive inhibition control (neutralizing antibody titer 64). The MARC-145 cells added with medium alone was use as negative control. The present of PRRSV in infected cells were determined by using immunoperoxidase monolayer assay (IPMA) at 3 days post infection as shown in **Figure 17**. Lower amount of PRRSV infected MARC-145 cells were found in cells treated with PEN-p-scFv no.5 and neutralizing swine serum when compare to control PEN-p-scFv, Poly:IC and positive infection.

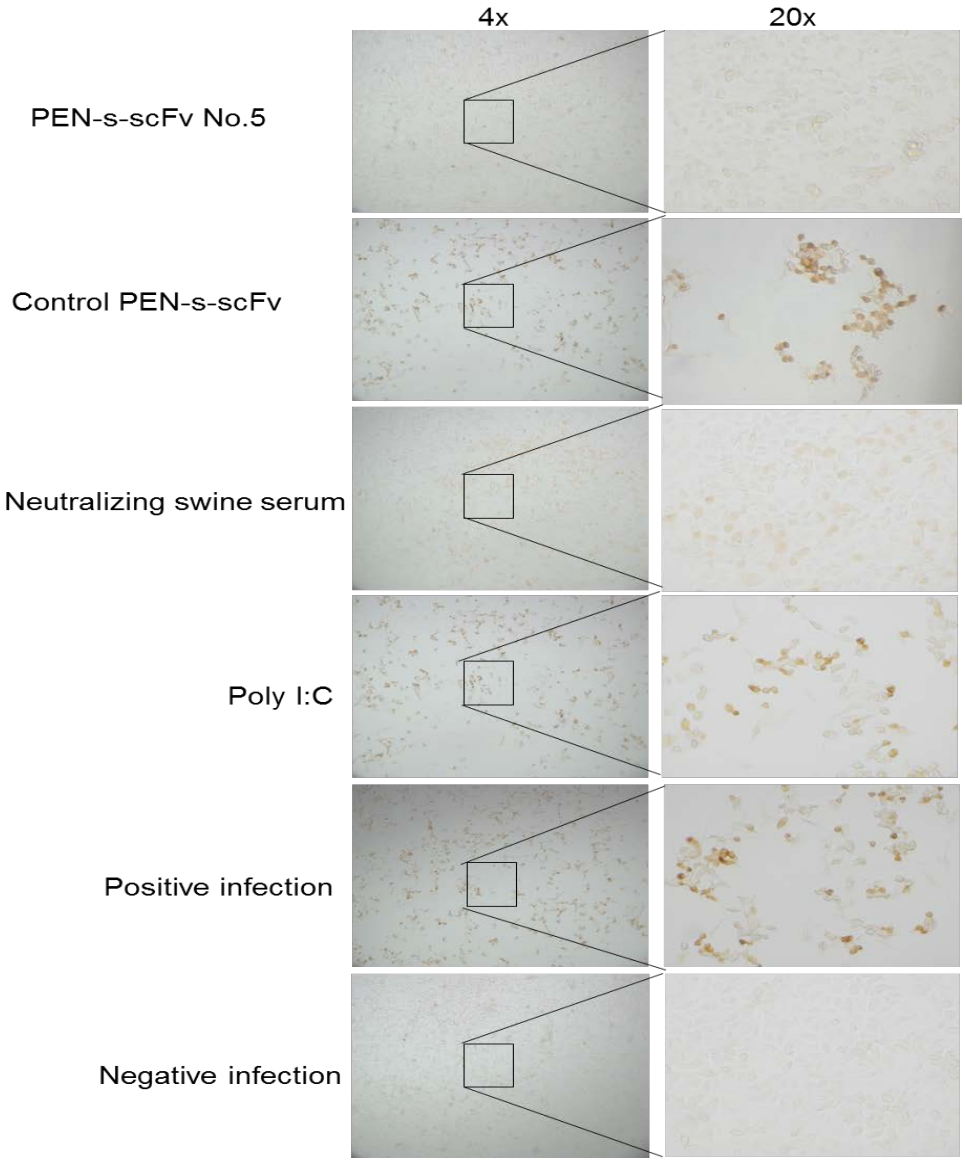

Figure 16 IPMA pattern of PRRSV infected MARC-145 cells treated with PEN-p-scFv no.5, control PEN-p-scFv, neutralizing swine serum, and Poly:IC, respectively.

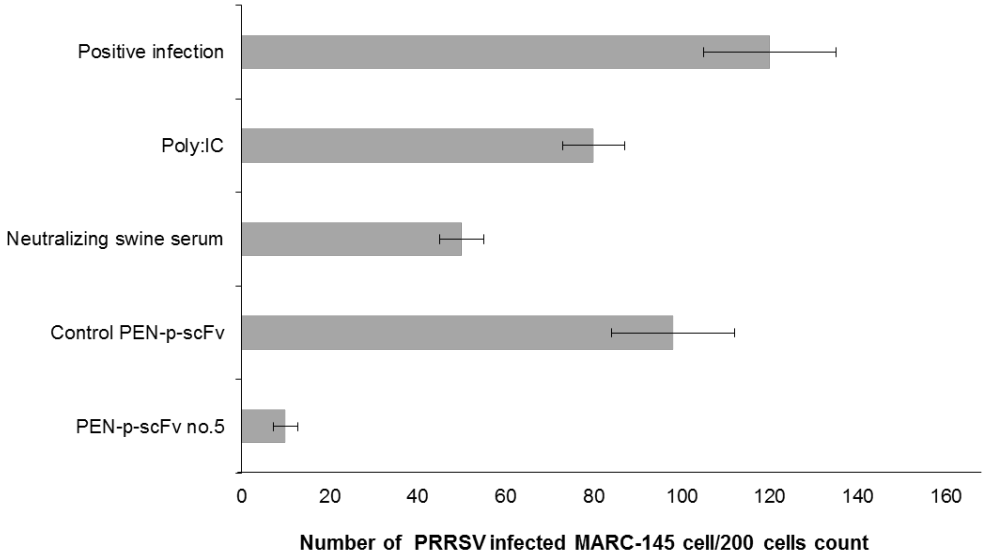

Figure 17 Number of PRRSV infected MARC-145 cell/200 cells count

Conclusion and Discussion

This study is the first study that try to produce porcine scFv phage display library in order to mimic antibody repertoire *in vitro*. These library can be used as a biological tool for the *in vitro* production of porcine antibodies of desired specificities. The p-scFv phage display library was successfully constructed. The PRRSV nsp1β recombinant protein was produced and used for selection specific p-scFv from the phage display library. The obtained cell penetrable porcine scFv (PEN-p-scFv) was produced because PRRSV nsp1β protein expressed in infected cells. The PEN-p-scFv has potential ability to interfere with PRRSV replication as shown that the infected MARC-145 cells treated with PEN-p-scFv clone no.5 has lowest infected foci when compared to control PEN-p-scFv, Poly:IC and neutralizing pig serum. However, the molecular of inhibition should be further study in order to understand for treatment remedy discovery.

Output

International Journal Publication: ongoing in process

Application: The data obtained from this study was used in class Veterinary clinical practice, Faculty of Veterinary, Kasetsart University, Bangkok, Thailand.