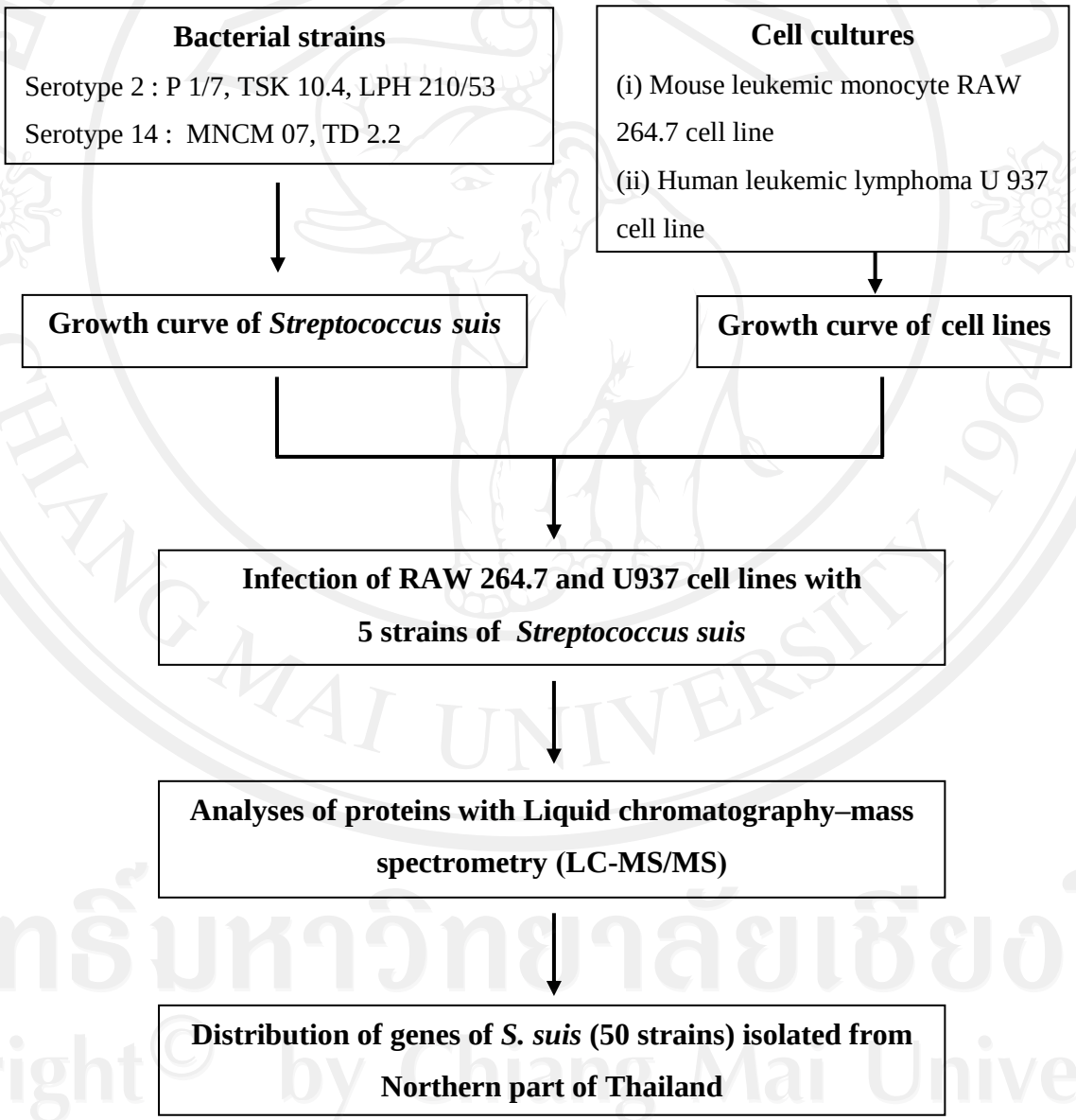
CHAPTER 4

Materials and methods

The experimental research plan



4.1 Bacterial strains and culture conditions

The *S. suis* serotype 2 and serotype 14 used in this study are listed in Table 4. Three strains of serotype 2 (P1/7; (reference strain); TSK 10.4; LPH210/53) were isolated from diseased pigs, healthy pig and patient, respectively. Two strains of serotype 14 (TD 2.2, MNCM07) were isolated from healthy pig and patient. Bacteria were cultured on blood agar plates, and a single colony was picked up and inoculated in Todd-Hewitt broth (THB) (Difco) at 37°C and 5% CO_2 until mid-log phase 3 to 5 h of incubation time. The culture was then adjusted to an optical density at 600 nm (OD_{600}) of 0.4 (about 10^8 to 10^9 CFU/ml) for further experiment.

Table 4 Origins of the *S. suis* serotype 2 and serotype 14 used in this study

Serotype	Strains	Specimen	Isolated from	Virulence-associated genes
2	P1/7 (Reference strain)	Ante-mortem blood culture from a pig dying with meningitis	Diseased pig	*sly*+/*epf*+/*mrp*+
	LPH 210/53	Hemoculture	Patient	*sly*+/*epf*+/*mrp*+
	TSK 10.4	Tonsil	Healthy pig	*sly*-/*epf*-/*mrp*+
14	MNCM 07	Hemoculture	Patient	*sly*+/*epf**+/*mrp*+
	TD 2.2	Tonsil	Healthy pig	*sly*+/*epf*+/*mrp*+

epf*, an *epf* variant that produces an ~3,000-bp fragment by PCR with primers described previously (Silva *et al*., 2006)

Growth of *Streptococcus suis*

A single colony of *S. suis* was picked and inoculated to 10 ml of THB and incubated for overnight at 37ºC with 5% CO_2. After that, the overnight culture was adjusted to OD_{600} = 0.05 (T_0) and cultured through a 12 hour incubation period. Culture medium was sampled at every 2 hours and growth of bacterial cultures was monitored by measuring the optical density at 600 nm. Number of viable cells were determined by plating onto THB agar.

4.2 Cell cultures

RAW 264.7 (Mouse leukemic monocyte) cell line was grown at 37ºC and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco).

U 937 (Human leukemic monocyte lymphoma) cell line was grown in RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), L-glutamine, and penicillin-streptomycin (Gibco).

Both cell lines were incubated at 37ºC in 5% CO_2 at a humid atmosphere and RAW 264.7 cell was split twice a week with 0.25% Trypsin-EDTA (1X) at a ratio of 1/3. Cells were used before 15^{th} passage for all experiments.

Growth study of cell lines

Cell lines (RAW 264.7, U937) were cultured in T-25 flask and incubated at 37°C with 5% CO_2 for overnight. Next day, cell lines were transferred to 24 wells plate (10^5 cells/well) and collected every day until about 7 to 8 days. The viability of cells was counted using 0.2% trypan blue (viable cell was colorless and death cell was blue) and the viable cell was calculated as follows:

% viability = (number of viable cells/total number of cells)×100

The cell numbers are counted as follows:

$$\text{Cells per ml} = \frac{\text{Total count x } 10^3}{0.4}$$

4.3 Cell invasion assay

The ability of each *S. suis* strain to invade cells was determined as previously described by Rubens, *et al.* 1992 and Lalonde, *et al.* 2000. Briefly, the mid-log phase culture (OD_{600} = 0.4) of each strains was centrifuged at 10,000 RPM for 5 min to harvest cells, washed twice with PBS (pH 7.2) and then resuspended as 5×10^7 CFU/ml in fresh cell culture medium without antibiotics.

Macrophage and monocyte cells (RAW 264.7 and U 937) were cultured in DMEM and RPMI 1640 (supplemented with 10% heat-inactivated FBS), respectively at 37°C and 5% CO_2 for 48 hours, and washed twice with PBS (pH 7.2). Cells were resuspended with cell culture medium contained 100 ng/ml phorbol myristate acetate (PMA) for cell stimulation (15-18 hours) before plated in six-well plate.

Bacterial suspension was added to wells containing a monolayer (about 10^6 cells) of macrophage cells in 1 ml medium at multiplicity of infection (moi) of 50 bacteria per cell in six-well plate. The plates were centrifuged at 800×g for 10 min and incubated for 2 hours at 37ºC with 5% CO_2. Cells were washed 3 times (2 ml per time) with PBS, and 2 ml of cell culture medium containing 100 µg/ml of gentamicin and 10 µg/ml penicillin G (Sigma) was added to each well to kill extracellular bacteria. After incubation for 2 hours at 37ºC with 5% CO_2, monolayers were washed 3 times with PBS and confirmed the efficiency of antibiotic treatment in killing extracellular bacteria by dropping a 10 µl sample of the last washing onto THB agar. After that, monolayers were incubated with 0.25% trypsin-EDTA (1X) 0.5 ml at 37ºC with 5% CO_2 for 10 min and added 10% of heat-inactivated FBS to stop the reaction of trypsin. Cells were lysed with 0.5 ml sterile distilled water and repeated pipetting to liberate intracellular bacteria. The 100 µl from each lysate was plated onto THB agar and incubated overnight at 37ºC with 5% CO_2. The rate of invasion was expressed as the total number of CFU recovered per well. The invasion assay was performed in duplicate and repeated at least three times.

4.4 Protein precipitation

Pellet of intracellular bacteria was resuspended with 0.5% SDS and added with two-fold cold acetone of 0.5% SDS. After incubation for overnight at -20 ºC, samples were centrifuged by two-step differential centrifugation to separate intracellular bacteria from host cell debris (Liu *et al*., 2012). Firstly, samples were pelleted at 1,500 rpm for 5 min to remove host cell nuclei, and then the post-nuclear supernatant was centrifuged again at 12,500 rpm for 20 min. The second pellets contained proteins of intracellular bacteria.

4.5 Protein determination

Bacterial proteins were resuspended in 0.15% Sodium Deoxycholic acid (DOC) and determined for protein concentration by Lowry method (Lowry et al., 1951). The absorbance at 750 nm (OD_{750}) was measured and the protein concentration was calculated using the standard curve, plotted between OD_{750} on Y-axis and BSA concentration (µg/ml) on the X-axis.

4.6 Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE)

The protein samples were separated by SDS-PAGE mini slab gel (8 x 9 x 0.1 cm, Hoefer miniVE, Amersham Biosciences, UK). The separating gel was prepared according to the standard method described by Laemmli (1970) with a 12.5% polyacrylamide gel. The equal volume of protein samples were mixed with 5 µl of 5X sample buffer (0.125M Tris-HCl pH 6.8, 20% glycerol, 5% SDS, 0.2M DTT, 0.02% bromophenol blue) and boiled at 95°C for 10 min before loading on the 12.5% SDS-PAGE. Determination of the molecular weight size of proteins was performed by using the standard marker (Amersham Biosciences, UK). Electrophoresis was performed in buffer (25mM Tris-HCl pH 8.3, 192mM glycine, 0.1% SDS) until the tracking dye reached the bottom of the gel and then gels were stained with silver staining (Blum, *et. al.*, 1987).

4.7 In-solution digestion

Protein samples were resuspended with 20 µl of 10 mM Ammonium bicarbonate before adding 5 µl of added 10 mM Dithiothreitol (DTT) in 10 mM ammonium bicarbonate and incubated at room temperature for 1 hour. The mixtures were then added with 20 µl of 100 mM Iodoacetamide (IAA) in 10 mM ammonium bicarbonate and further incubated at room temperature for 1 hour in the dark. After that, the samples were added 5 µl of trypsin solution (10 ng Trypsin in 50% acetronitrile/10 mM ammonium bicarbonate) and incubated at room temperature for 1 to 3 hours. The peptide samples were then dried with SpeedVac for 1 to 2 hours before resuspending with 10 to 15 µl of 0.1% trifluoroacetic acid (FA) for analysis by Liquid chromatography mass spectrometry (LC-MS/MS).

4.8 In-gel digestion

Protein samples were separated by molecular weight with SDS-PAGE electrophoresis and different bands of proteins (about 15-20 bands) were selected for digestion. Gel plugs were excised into small ($1\times1\times1$ mm^3) cubes and transferred into 96 wells plate (5 gel plugs/well). Gel plugs were washed with sterile distilled water 200 µl and shaking on Microtiter plate shakers at room temperature for 5 min, dehydrated with 200 µl of 100% acetronitrile (ACN), 5 min for 3 times. After that, gel plug were dried at room temperature for 5 to 10 min, before added 50 µl of 10 mM Dithiothreitol (DTT) in 10 mM ammonium bicarbonate and incubated at room temperature for 1 hour to reduced peptide bonds of proteins. To prevent disulfide bond formation, 50 µl of 100 mM Iodoacetamide (IAA) in 10 mM ammonium bicarbonate was added and the mixture was incubated at room temperature for 1 hour in the dark for alkylation. The gel pieces were then dehydrated with 200 µl of 100% acetronitrile (ACN) for 5 min (2 times) and then added with 20 to 40 µl of trypsin solution (10 ng Trypsin in 50% acetronitrile/10 mM ammonium bicarbonate) and incubated at room temperature for 20 min. Next, the gels were immersed throughout digestion by adding 30 µl of 30% acetronitrile and incubated at room temperature for 3 hours or overnight before transferring to new microtiter plate. Gel samples were extracted with 30 µl of 50% acetronitrile in 0.1% formic acid (FA) and incubated at room temperature for 10 min. The gel samples were then dried in hot air oven at 40 ^{o}C for 3 to 4 hours or overnight. Peptides were collected and pooled together in the new microcentrifuge tube (1.5 ml) and centrifuged at 10,00 rpm for 5 min. The 8 µl of extracted peptides were pipetted to insert a tube for protein identification by LC-MS/MS.

4.9 Liquid chromatography mass spectrometry (LC-MS/MS) analysis

Peptide digests were injected by HCTultra PTM Discovery System (Bruker Daltonics Ltd., U.K.) coupled to UltiMate 3000 LC System (Dionex Ltd., U.K.). Peptides were separated with nanocolumn (PepSwift monolithic column 100 m i.d. x 50 mm). Mobile phase used was composed of solvent A, which was 0.1% formic acid (FA) in water and solvent B, which was 80% acetonitrile in water containing 0.1% formic acid. Peptide fragment mass spectra were acquired in data-dependent AutoMS (2) mode with a scan range of 300−1500 *m*/*z*, 3 averages, and up to 5 precursor ions selected from the MS scan 50−3000 *m*/*z*.

Peptide peaks were detected automatically using Data Analysis version 4.0 (Bruker). Mass lists in the form of Mascot generic files were created automatically and used as the input for Mascot MS/MS Ions searches of the National Center for Biotechnology Information nonredundant (NCBI nr) database (www.matrixscience.com). (Parameters for search: Enzyme = trypsin, max. missed cleavages =1; fixed modifications = carbamidomethyl (C); variable modifications = oxidation (M); peptide tolerance ±1.2 Da; MS/MS tolerance ±0.6 Da; peptide charge = 1+, 2+ and 3+; instrument = ESI-TRAP).

4.10 Protein identification

Identification of proteins by peptide-mass fingerprinting were performed by using DeCyder MS Differential Analysis software (DeCyderMS, GE Healthcare (Johansson *et al*., 2006; Thorsell *et al*., 2007) for proteins quantitation. The MS/MS data was submitted to database search with the Mascot software (Matrix Science, London, UK, (Perkins *et al*., 1999)) for data analysis. Proteins of interest should have more than two peptides with an individual mascot score corresponding top<0.05 and p<0.1, respectively.

4.11 DNA extraction

S. suis strains were cultured in THB at 37ºC with 5% CO_2 for overnight. The 1 ml of overnight bacterial culture was transferred in to a 1.5 ml microcentrifuge tube and centrifuged at 10,000 RPM for 5 min to harvest cells. Briefly, the DNA isolation was performed by adding 40 µl of lysozyme buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 0.1 M NaCl, 5% Triton X-100) to bacterial cells and immediately mix by vortex. Then, 10 µl of lysozyme (10 mg/ml in 10 mM Tris-HCl, pH 8.0) was added to sample and incubated at 37ºC for 15 min. After that, 20 µl of proteinase K (20 mg/ml) was added, mixed for vortex 30 sec, and incubated at 55 ºC for 10 to 15 min in a water bath. Then, 500 µl of lysis buffer (BD buffer) was added to the sample and incubated at room temperature for 5 min. Two-fold absolute ethanol (v/v) was added to the sample before application each sample to a separate column. The column was then spinned for 1 minute at 1,100 rpm, the flowthrough was discarded. DNA was washed twice with 500 µl of wash buffer and centrifuged for 5 minutes at 10,000 rpm. DNA sample was then eluted with 200 µl of pre-heated elution buffer and incubated at room temperature for 2 min before centrifuging for 5 to 10 min at 10,00 rpm (2 times) to recover the purified genomic DNA as flowthrough. The purified genomic DNA was stored at -20 ºC.

4.12 Multiplex-PCR

Genomic DNA of *S. suis* was used as template for M-PCR amplification using 3 pairs of specific primers of virulence associated genes (extracellar factor protein: *epf*, suilysin: *sly* and muramidase released protein: *mrp*) as shown in Table 5 (Silva *et al.* 2006). The PCR components contained 5.0 µl of 5X MyTaq^{TM} Reaction buffer (5 mM dNTPs and 15 mM $MgCl_2$), 1.1 µl of all primer 10 µM of forward primers and reverse primers (*sly*_F (0.2 µl), *epf*_F (0.15 µl), *mrp*_F (0.2 µl), *sly*_R (0.2 µl), *epf*_R (0.15 µl), and *mrp*_R (0.2 µl)), 0.25 µl of *Taq* DNA polymerase^{TM} (5 unit/µl), 1.0 µl of the DNA template and 17.65 µl of deionized water in a total volumne of 25 µl. Deionized water was used to replace DNA template for the negative control. The amplification was performed for 35 cycles of PCR process under the following thermal cycling condition: the initial denaturation at 94 ºC for 2 minutes, the denaturation at 94 ºC for 1 minute,

the annealing at 58 ºC for 1 minute, the extension at 72 ºC for 1.30 minutes, and the final extension at 72 ºC for 2 minutes prior to holding at 10 ºC. All PCR products were analysed by electrophoresis in 1.0% agarose gel, stained with 0.5 µg/ml ethidium bromide, and visualized under UV light (Gel doc). DNA ladder markers®Thermo scientific , 100 bp Plus were used to access the band size.

Table 5 Oligonucleotide primers of virulence-associated genes

PCR assay	Gene	GenBank accession number	Primer sequence (5'-3')	Position in coding sequence	PCR product size (bp)	Reference
Multiplex	*epf*	X71881	CGCAGACAACG AAAGATTGA AAGAATGTCTT TGGCGATGG	2040-2059[a], 2764-2783[a]	744[a]	Silva *et al.* 2006
	sly	Z36907	GCTTGACTTAC GAGCCACAA CCGCGCAATAC TGATAAGC	116-135, 345-363	248	Silva *et al.* 2006
	mrp	X64450	ATTGCTCCACA AGAGGATGG TGAGCTTTACC TGAAGCGGT	3478-3497[a], 3646-3665[a]	188	Silva *et al.* 2006

epf: extracellar factor protein, ***sly***: suilysin, ***mrp***: muramidase released protein

[a] Position in the gene and PCR product size in EF and MRP reference strain D282

Table 6 *Streptococcus suis* strains used in the distribution study

Isolate no.	Site of isolation	Diseases and symptoms	ST [b] (ST complex)
MNCM 01	Blood	Endocarditis	1 (1)
MNCM 06	Blood, CSF	Neck stiffness, deafness (meningitis)	1 (1)
MNCM 16	CSF	Neck stiffness (meningitis)	1 (1)
MNCM 21	CSF	Meningitis	101 (27)#
MNCM 25	Blood	Neck stiffness (meningitis), diarrhea, death	102 (27)#
MNCM 26	Blood	Endocarditis, deafness (meningitis)	25 (27)#
MNCM 43	Blood	Endocarditis	28 (27)
MNCM 50	Blood	Pulmonary edema, death	104 (27)#
MNCM 51	Blood	Septicemia, diarrhea, death	25 (27)#
MNCM 54	Blood	Neck stiffness (meningitis), diarrhea	102 (27)#
MNCM 55	Blood	Septic shock, death	25 (27)#
LPH 03	Blood	Meningitis	103 (27)#
LPH 04	Blood	Septicemia, diarrhea	25 (27)#
LPH 05	Blood	Septicemia	103 (27)#
LPH 12	Blood	Septic shock, death	25 (27)#
H 131/53	Blood	Septicemia	ND
H 132/53	Blood	Septicemia	ND
H 148/53	Blood	Septicemia	ND
H 153/53	Blood	Septicemia	ND
H 187/53	Blood	Septicemia	ND
H 219/53	Blood	Septicemia	ND
H 240/53	Blood	Septicemia	ND
H 244/54	Blood	Septicemia	ND
H 286/54	Blood	Septicemia	ND

Table 6 (Continued)

Isolate no.	Site of isolation	Diseases and symptoms	ST [b] (ST complex)
H 290/54	Blood	Septicemia	ND
LDK 19.2	Tonsil	None	ND
LSK 15.2	Tonsil	None	ND
T 11.3 N	Tonsil	None	ND
T 13.1 N	Tonsil	None	ND
T 16.2 N	Tonsil	None	ND
T 6.1 S	Tonsil	None	ND
TD 2.3	Tonsil	None	ND
TDK 5.4	Tonsil	None	ND
THD 10.2	Tonsil	None	ND
THD 10.4	Tonsil	None	ND
THD 10.6	Tonsil	None	ND
THD 16.4	Tonsil	None	ND
TJ 21.2 S	Tonsil	None	ND
TP 12.2	Tonsil	None	ND
TP 12.3	Tonsil	None	ND
TSK 11.2	Tonsil	None	ND
TSK 16.2	Tonsil	None	ND
TSK 16.3	Tonsil	None	ND
TSK 16.4	Tonsil	None	ND
TSP 19.2	Tonsil	None	ND

[b] ST, sequence type; ND, Not determined