CHAPTER III

MATERIALS AND METHODS

Virus

The CSFV used for challenge was the Thai isolate, high virulent strain (Bangkok 1950 strain) obtained from National Institute of Animal Health (NIAH, DLD). The virus was titrated to have a titer of $10^5 TCID_{50}$/ml and kept at -70°C until use.

Animals

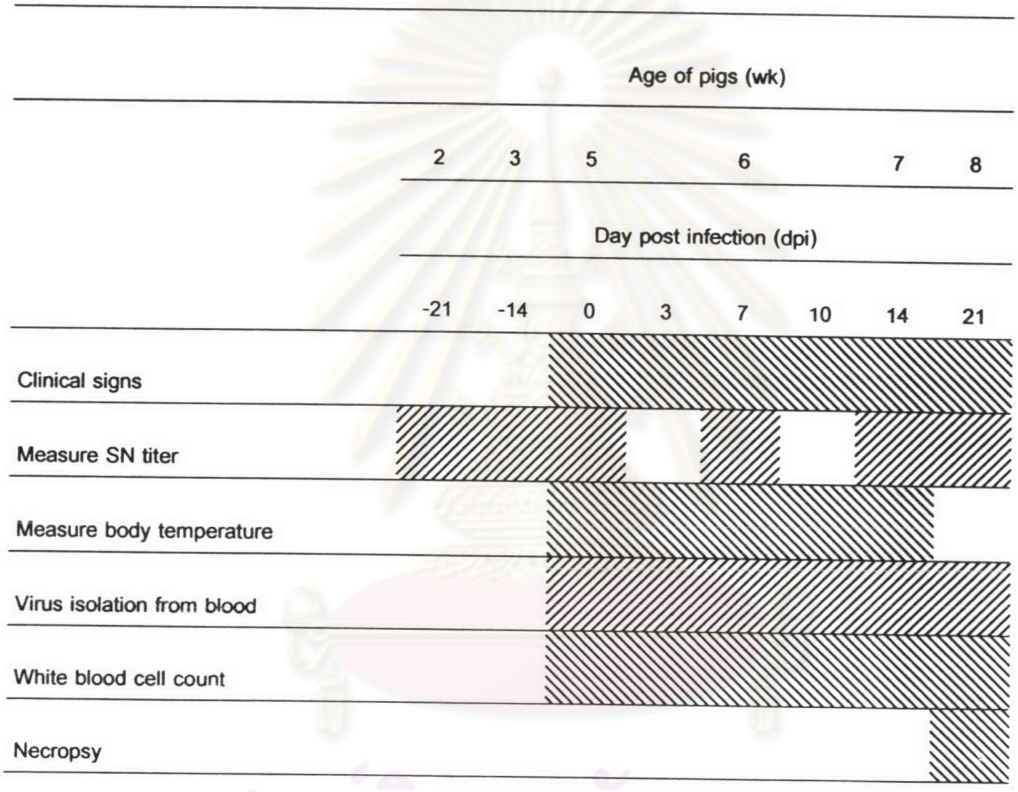
Piglets, cross bred (Landrace x Largewhite x Duroc), 2 weeks of age, were purchased from a conventional farm which proved to be free from CSF during the past 2 years. The pigs were maintained with food and water provided *ad libitum*.

Experimental Designs

The blood samples were collected at the day of entry for measuring the antibody titers against CSFV. The pigs were divided into to 3 groups (6 animals of each), A, B, and C according to the level of passive neutralizing antibody (SN) titer at the day of vaccination as high ($\geq$ 64), low ($\leq$ 8), and wide range from $\geq$ 64- $\leq$ 8 (control group), respectively. At 3 weeks of age, serum samples were then collected to measure their passive SN titers. Pigs in group A and B were vaccinated intramuscularly with lapinized CSFV vaccine (Chinese strain) obtained from NIAH. On the 14th day post-vaccination (dpv), all pigs including pig of group C were challenged intramuscularly with CSFV at $5x10^5 TCID_{50}$/pig. Clinical signs and rectal temperature were assessed daily for 2 weeks after challenged. Whole blood were collected on 0, 3rd, 7th, 10th, 14th, and 21st day post-infection (dpi) for white blood cells count using automatic counter (BAKER 150, Serono Baker Diagnostis, USA.) and CSFV detection using immunoperoxidase monolayer assay (IPMA) (Terpstra, 2000). Sera were taken on 0, 7th, 14th, and 21st dpi

and tested for SN titer against CSFV using NPLA. At the end of the experiment, on the 21st dpi, all animals were euthanized for necropsy (Table 1).

Table 1 Time table for clinical observation and laboratory investigation for experimental pigs. The pigs were vaccinated with CSFV-vaccine (lapinized Chinese strain) at 3 week of age and challenged at 5 week of age (n=6).



Post Mortem Examination

All animals were euthanized by pentobarbital sodium and saturated magnesium sulfate. Complete necropsy was performed. Organ samples, especially lymphoid tissues (thymus, tonsil, spleen, ileum, lymph nodes) were fixed in 10% neutral buffered formalin for microscopic examination. All samples were conventionally processed and embedded in paraffin wax. Sections were cut for immunohistochemistry and stained with haematoxylin and eosin (HE) for histopathological examination. Depletion of lymphoid tissues were observed and scored according to the following

criteria: severe depletion, all or most of lymphoid follicles were absent (+++), moderate depletion, there were multifocal necrosis and reduced number of lymphoid follicles about 50% (++), mild depletion, some areas of lymphoid tissues were thin but the follicles remain (+), and negative (-), no remarkable lesion in lymphoid tissues.

Virus Isolation

Virus isolation from whole blood was performed by IPMA (Terpstra, 2000), the dilutions of 50 µl of blood samples were added on to preform monolayer of PK15 cells at final volume 100 µl/well. After an incubation period of 4 days at 37°C in a 5% CO_2 atmosphere, the plates were briefly rinsed with saline, drained and fixed with 4% formalin in phosphate buffered saline plus Tween-20 (PBST) 100 µl/well for 20 min at room temperature (RT), rinsed three times with PBST 150µl/well. The monolayer was stained by immunoperoxidase.

Immunoperoxidase were performed by overlaid the monolayer with 50µl of mouse anti-CSFV gp55 MAb (provided by NIAH) then incubated at RT for 1 hr. Subsequently, the plates were washed three times with PBST and 50µl of commercially goat anti-mouse IgG horseradish peroxidase conjugate (DAKO, Glostrup, Denmark), diluted at 1/300 was added to each well, and incubated at RT for 1 hr. The washing process was performed and the substrate of the enzyme, 0.4% 3-amino-9-ethyl-carbazole (AEC, Sigma-Aldrich, MO, USA) in acetate buffer and 30% H_2O_2 in the ration of 1ml: 19ml: 20 µl was added 100 µl/well. After 1 hr of incubation at RT, the reaction was stopped by discarded the fluid and then washed with distilled water (DW). The presence of CSFV was indicated by the characteristic of carmine-red color in the infected cells.

Determination of Serum Neutralizing Antibody to CSFV

Serum neutralizing antibody titers to CSFV were determined by NPLA (Pachariyanon et al., 1997). The sera were first inactivated at 56°C for 30 min to destroy heat-labile nonspecific virus inhibitory substances. Then, the serum samples were

diluted serial two fold with MEM (add fetal calf serum to final concentration of 5%) in 96 wells tissue culture plate. Add 50µl of CSFV (ALD strain, provided by NIAH) suspension to the wells, diluted in MEM to contained approximately 200 $TCID_{50}$/50 µl. Incubate the plates for 1 hr at 37°C in a 5% CO_2 incubator. After that 100 µl of cells suspension were added to each well. Allow the cells to grow at 37°C in 5% CO_2 to become confluent for 4 days and then the plates were rinsed briefly with saline, drained and fixed with 4% formalin in PBST (100 µl/well) for 20 min at RT, rinsed three times with PBST (150 µl/well). The monolayer was stained by immunoperoxidase as described above. The test was read visually or microscopically using inverted microscope. Infected cell sheets were indicated as completely or partially stained reddish brown. SN titer was the reciprocal of the highest dilution of serum sample that completely inhibit virus infection in the cell culture.

Detection of CSFV Ag in Lymphoid Tissues by Immunohistochemistry

A. Tissues preparation

Lymphoid tissues, thymus, tonsil, spleen, ileum, spleen, and lymph node were histopathological prepared for the detection of CSFV Ag and apoptosis. Four micrometer thickness of formalin-fixed paraffin-embedded tissues were cut and placed on poly-L-lysine coated slide. The slides were incubated in hot air oven at 60°C for 30 min and immediately deparaffinized in xylene twice for 10 min each and then absolute ethanol 2X5 min each at RT, followed by gradually diluted ethanol in distilled water at 95%, 80%, 70%, and 50% 1 min each dilution, respectively, and finally in DW.

B. Antigen retrieval technique

IHC sensitivity was enhanced by heat induced epitope retrieval. The sections were submerged in 0.1 M Tris buffer, pH 7.5 with 0.125% Tween-20 and then autoclaved at 121°C, 2 *psi*/inch^2 for 10 min and then cool down until the buffer reach 40°C. The sections were washed with DW and PBS twice 3 min each.

C. Immunohistochemical procedure

Endogenous peroxidase activity in tissue sections was eliminated by submerge tissues in 0.3% H_2O_2 in methanol for 30 min at RT and washed once with DW and PBS 3x3 min each. Blocking of non-specific reactions was done by applying 2% of bovine serum albumin (BSA, Sigma-Aldrich) in PBS in humidified chamber at 37°C for 30 min and then washed with PBS 3x3 min each. The primary antibody, mouse anti-CSFV gp55 MAb (clone A18-24/10, Weybridge, UK) was applied at 1:50 and then incubated in humidified chamber at 4°C for 12 hr, washed with PBS 3x5 min each. The biotinylated rabbit anti-mouse IgG antibody (1:400, Kirkegaard & Perry Laboratories; KPL, MD, USA) was added and incubated in humidified chamber at 37°C for 30 min and then washed with PBS 3x3 min each. Applied avidin-biotin complex peroxidase solution (ABC kit, DAKO, Denmark) was used and incubated in humidified chamber at RT for 30 min and then washed with PBS 3x3 min each. The colorization was done by dipping in 0.5% 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) in 0.05 M Tris-HCl and 0.3% H_2O_2 for 5 min or until well-developed brown color were observed and the reaction was stopped by DW. Sections were counterstained with methyl green and visualized by light microscope.

In situ Detection of Apoptosis of Lymphoid Tissues

A. Tissues preparation, antigen retrieval technique, and elimination of endogenous peroxidase activity.

The lymphoid tissues were prepared by the similar methods as previously mentioned for the detection of CSFV-Ag step A&B.

B. *In situ* DNA fragmentation detection method

Apoptosis detection of lymphoid tissues was done using ApopTag®, *in situ* apoptosis detection kit (Intergen Co., MA, USA.) based on modified TUNEL assay (Gavrieli et al., 1992). The treated tissues were immediately applied by equilibration buffer and incubated at RT for at least 5 min and then pipette onto the section of working

strength TdT enzyme and incubated in humidified chamber at 37°C for 1 hr. The section was put in a coupling jar containing working strength stop solution, agitated for 15 sec, and incubated at RT for 10 min, then washed the specimen with PBS for 3x1 min. The anti-digoxigenin peroxidase conjugate antibody was applied to the tissues and incubated in a humidified chamber at RT for 30 min and wash the sections in PBS for 4x3 min. The peroxidase-diaminobenzidine substrate was completely applied to cover the sections and incubated at RT for 3-7 min until the color was developed. In order to determine the optimal staining time the color development, was monitored by observing under the microscope. The color reaction was stopped by washing the slides with DW for 3x1 min and then leaving for 5 min. Finally, the sections were counterstained with 0.5% (w/v) methyl green for 10 min at RT and then washed in DW for 3x1 min and 3 change of absolute ethanol by dipping 10 times each. The sections were mounted with permount and visualized under light microscope.

Agarose Gel Electrophoresis for the Detection of DNA Ladder Formation of Apoptosis in Lymphoid Tissues

A. DNA extraction

Formalin-fixed, paraffin-embedded tissues of tonsil, thymus, spleen, lymph nodes, and Peyer's patches were selected to detect fragmentation of DNA using the method adapted by Gavrieli et al. (1992). The tissues were cut at 10 µm, 10 sections, deparaffinized in 3 changes of xylene 10 min each and then 3 changes of absolute ethanol 10 min each. The tissues were dried at 60°C and suspended in 1 ml of DNA extraction buffer (Appendix C) then the suspensions were incubated at 56 °C in shaking incubator for 24 hr. During the incubation period, 10 µl of fresh proteinase K (20 mg/ml) was added every 6 hr. DNA was extracted by using phenol : chloroform : Isoamyl alcohol (25 : 24 : 1) method. Add an equal volume of phenol-chloroform to 500 µl of the suspension then the suspension was gently mixed for 10 min and then centrifuged at 3000 rpm for 5 min. An aqueous fraction, the top layer, was collected to a new tube. Add an equal volume of the phenol-chloroform to the aqueous solution and

repeated the procedures described above 2-3 cycles. The top layer was precipitated with 0.3 M sodium acetate and cool absolute ethanol (on ice). The mixture was allowed on ice for 30 min for complete precipitation of DNA which then pelleted by high speed centrifugation (14,000 rpm) at 4°C for 30 min. The DNA pellet was washed by 70% ethanol and dried at 37°C for 30 min then dissolved in 100 µl of Tris-EDTA buffer (TE) pH 8.0

B. Agarose gel electrophoresis

DNA samples were electrophoretically separated on 3% agarose (GENE Pure™ HiRes, ISC BIOEXPRESS, Spain) in Tris-Boric acid-EDTA solution (TBE) using eletrophoretic chamber (Pico-2, TAITEC, Japan) with 80 volt for 2 hr. After that, the gel was stained with 0.5 µg/ml of ethidium bromide (Sigma Chemical Co.) for 20 min and visualized and photographed with a UV illuminator (VILBER LOURMAT, France).

Quantification of CSFV-infected Cells and Apoptotic Cells in Lymphoid Tissues

Method for evaluate CSFV infected cells and apoptotic cells was made by a little modification of criteria in the study of Resendes et al. (2004). The section was positioned under the microscope using X10 ocular and X20 objective, X400 of magnification that was approximately calculated as 0.1 mm^2/field. The microscope fields were randomly positioned 10 fields/ section. Every positive cell was counted and expressed as the mean of positive cells/field/section.

Data Analysis

Statistical analyses for the SN titer, apoptotic cells and CSFV-infected cells were performed by ANOVA using the SPSS procedures (SPSS Inc., USA). ANOVA was applied for analysis of SN titer, of pigs in each group at various dpi. The significance of differences of means among groups at each dpi was further assessed by LSD and the level of significance was set at $P<0.05$. For analysis of the apoptotic and CSFV-infected cells, T-test was applied for each group and tissue type. Measure of

association between the apoptosis and infected cells was done by correlation coefficient.