CHAPTER 2

MATERIALS AND METHODS

1. Materials

1.1 Organisms

1.1.1 Bacillus anthracis

Two strains of B. anthracis were employed in this project. The uncapsulated avirulent strain $34F_2$ that originally isolated by Sterne was used for producing living spore vaccine and the virulent strain that was sporadic in Thailand was used as challenged strain.

1.1.2 Pasteurella multocida

The organism was type 6:B that was endemic in Thailand.

The organisms above were obtained from the Division of Veterinary Biologics, the Department of Livestock, Ministry of Agriculture and Cooperatives.

1.2 Experimental animals

1.2.1 Rabbits: adult New Zealand rabbits weight about 2.4 to 2.8 kg were used.

1.2.2 Mice: young, white, male Swiss mice, weight about 18 to 20 g were used in this study.

1.3 Media

1.3.1 Bacto-Beef extract (Difco Laboratory, U.S.A.)

1.3.2 Bacto-Fluid Thioglycollate Medium (Difco Laboratory, U.S.A.)

1.3.3 Bacto-Peptone (Difco Laboratory, U.S.A.)

1.3.4 Bacto-Tryptose Agar (Difco Laboratory, U.S.A.)

1.4 Chemicals

1.4.1 Albumin bovine, crystallized (Sigma Chemical Company)

1.4.2 Aluminum hydroxide gel

1.4.3 Ammonium sulfate (May and Baker Ltd., England)

1.4.4 Disodium hydroxide phosphate (May and Baker Ltd., England)

1.4.5 Folin Ciocalteu's phenol reagent (BHD. Chemical Ltd., England)

1.4.6 Formaldehyde 40% w/v (Carlo Erba., Italy)

1.4.7 Glycerin (BHD. Chemical Ltd., England)

1.4.8 Sodium chloride (BHD. Chemical Ltd., England)

1.4.9 Sodium dihydrogen phosphate (May and Baker Ltd., England)

1.4.10 Sodium hydroxide (May and Baker Ltd., England)

1.4.11 Xylene (Mallinckrodt

1.5 Glasswares

1.5.1 Beakers (Pyrex, U.S.A.)

1.5.2 Bright Line Haemacytometer (American Optical Co.,U.S.A.)

1.5.3 Burette

1.5.4 Centrifuge tubes

1.5.5 Erlenmeyer flasks (Pyrex, U.S.A.)

1.5.6 Funnels (Pyrex, U.S.A.)

1.5.7 Glass beads

1.5.8 Glass slides (Clay Adams, U.S.A.)

1.5.9 Measuring cylinders (Pyrex, U.S.A.)

1.5.10 Pasteur pipettes

1.5.11 Petri dishes (Pyrex, U.S.A.)

1.5.12 Pipettes (Pyrex, U.S.A.)

1.5.13 Roux bottles

1.5.14 Serological test tube (Pyrex, U.S.A.)

1.5.15 Stirring rod

1.5.16 Syringe (Fortuna, Western Germany)

1.5.17 Test tubes (Pyrex, U.S.A.)

1.5.18 Weighting bottles

1.6 Instruments

1.6.1 Analytical balance H6T (E. Mettler, Switzerland)

1.6.2 Autoclave model HA-3D (Hirayama Manufacturing Corporation, Japan)

1.6.3 Centrifuge (Clay Adams)

1.6.4 Colony counter (New Brunswick Scientific, U.S.A.)

1.6.5 Deep freeze refrigerator (Continental)

1.6.6 Dialysis tubing (Union Carbide Corp.)

1.6.7 Hot plate (Chromalox, U.S.A.)

1.6.8 Incubator, Precision model 6 (Precision Scientific Co., U.S.A.)

1.6.9 Light microscope (Olympus, Japan)

1.6.10 Oven, Precision Model 27 (Precision Scientific Co., U.S.A.)

1.6.11 Refrigerator (Marco)

1.6.12 Magnetic stirrer (Mag-Mix) (Precision Scientific Co., U.S.A.)

1.6.13 Spectronic 710 (Bausch & Lomb, U.S.A.)

1.6.14 Vortex cyclomixer (Clay Adams, U.S.A.)

1.6.15 Water bath (Precision Scientific Co., U.S.A.)

1.7 Others

1.7.1 Filter paper (Whatman Limited, England)

1.7.2 Hypodermic needles (K51, Italy)

1.7.3 Pinted scissors (A Dumont & Films)

1.7.4 Sheep blood

1.8 Preparation of solution

1.8.1 Saline solution (0.85%)

Sodium chloride	8.5	g
Distilled water to	1000.0	ml

1.8.2 40% glycerin saline

Glycerin	40.0	ml
Saline solution to	100.0	ml

1.8.3 0.2% formalinized saline

Formaldehyde	0.2	ml
Saline solution to	100.0	ml

1.8.4 Saturated ammonium sulfate solution

Ammonium sulfate	1000.0	g
Distilled water	1000.0	ml

Dissolved 1000 g ammonium sulfate in 1000 ml distilled water at 50°C, allowed to stand over night at room temperature and adjusted the pH to 7.2 with dilute ammonium solution or sulfuric acid before used.

1.8.5 Phosphate-buffer saline (PBS)

(0.01 M Phosphate in saline, pH 7.0)

Solution A (NaH_2PO_4, 0.2 M)

Sodium dihydrogen phosphate ($NaH_2PO_4 \cdot 2H_2O$)	31.2	g
Distilled water to	1000.0	ml

Solution B (Na_2HPO_4, 0.2 M)

Disodium hydrogen phosphate (anhydrous)	28.39	g
Distilled water to	1000.0	ml

Dissolved each stock solution seperately and stored at room temperature. To prepare 1-liter of 0.01 M PBS, delivered the stock solution in the following amounts:

Solution A	16.5	ml
Solution B	33.5	ml
Sodium Chloride	7.4	g
Distilled water to	1000.0	ml

2. Methods

2.1 Preparation of vaccines

2.1.1 Preparation of monovalent vaccines[88]

2.1.1.1 *Bacillus anthracis* vaccine or anthrax spore vaccine

To prepare seed material for the Roux bottles, an ampule of the dried avirulent culture (Strain $34F_2$) was opened aseptically, the content was suspended in less than a milliliter of nutrient broth and then seeded on solid medium. After 24 hr incubation at 37°C, the culture was inoculated in 200 ml nutrient broth and incubated 17-18 hr at 37°C. The growth was checked for purity.

This allowed 3-4 ml to inoculate on 300 ml Difco tryptose agar in Roux bottle and then incubated for three days at 37°C after which time the bottle stored for another 10 days in the dark at room temperature that sporulation was satisfactory (80-90%).

The sporulated growth was washed off with sterile saline solution and suspended in twice the volume of glycerin. After a period of not less than three weeks in dark at room temperature the concentration of viable spores in the suspension was estimated by plate counts and diluted to contain 1×10^7 viable spores per ml with 40% glycerin saline.

2.1.1.2 Pasteurella multocida vaccine or hemorrhagic septicemia vaccine

The seed material prepared quite simply as method described in 2.1.1.1

The seed was inoculated on 300 ml Difco tryptose agar in Roux bottle. After 24 hr incubation at 37°C, the growth was washed off with 0.2% formalin. This formalin-killed bacterial suspension was diluted to contain 1 x 10^9 cells per ml. After further incubation for a period of 24 hr , this was tested for sterility in fluid-thioglycollate media. Any contamination was discarded.

The 2% aluminum hydroxide gel was added to formalin-killed bacterial suspension to make final 1% aluminum hydroxide in alum-precipitated (hemorrhagic septicemia) vaccine so the vaccine contained 0.5 x 10^9 cells per ml.

2.1.2 Preparation of bivalent vaccine of B. anthracis and P. multocida

The following two groups of bivalent vaccine were prepared immidiately before immunization in rabbits.

2.1.2.1 Bacillus anthracis vaccine combined with alum-precipitated vaccine of P. multocida

Five milliliters of Bacillus anthracis vaccine (2.1.1.1) were added to 10 ml of alum-precipitated vaccine (2.1.1.2) and the two were mixed well.

2.1.2.2 Bacillus anthracis vaccine combined with formalin-killed culture of P. multocida

Five milliliters of Bacillus anthracis vaccine (2.1.1.1) were added to equal volume of formalin-killed bacterial suspension (2.1.1.2) and the two were mixed well.

2.2 Rabbit immunization

2.2.1 Production of antiserum[89]

Twelve rabbits were divided equally into four groups. Each group was bled before immunization of each vaccine in 2.1 was applied as following.

2.2.1.1 Bacillus anthracis vaccine was administered subcutaneously on the abdomen in amounts of 0.5 ml per dose at day 0, 5, 10 and followed intravenously in the same dose at day 15 and day 20 to each of three rabbits.

2.2.1.2 The alum-precipitated vaccine of P. multocida was administered subcutaneously on the abdomen in amounts of 1.0 ml per dose at day 0, 5, 10 and then formalin-killed bacterial suspension was administered intravenously in amounts of 0.5 ml per dose at day 15 and day 20 to each of three rabbits.

2.2.1.3 Bivalent vaccine with aluminum hydroxide gel in 2.1.2.1 was immunized the rabbits by the same procedure as 2.2.1.2. The combined vaccine with alum was administered subcutaneously in three seperated doses of 1.5 ml each at day 0, 5, 10 and then the combined vaccine without alum in 2.1.2.2 was

administered intravenously in amounts of 1.0 ml per dose at day 15 and day 20 to each of three rabbits.

2.2.1.4 Bivalent vaccine with aluminum hydroxide gel in 2.1.2.2 was immunized the rabbits by the same procedure as 2.2.1.1. The combined vaccine without alum was administered in five seperated doses of 1.0 ml each.

2.2.2 Collection of antiserum[90]

Serum samples were collected from each group of rabbits prior to each immunization.

Ten days after the last injection, trial bleeding was made to determine sufficient antibody. When antibody titer was satisfactory, the heavy bleedings were made by drawing the blood five times, about 40 ml each, at two days intervals. The several serum collections were worked up seperately and could be pooled in each group of vaccines. The antiserum were stored at $4^{o}C$. These were used in passive protection tests.

After the heavy bleedings, each group of immunized rabbits were further bled in about weekly intervals within seven months to determine the level of immunity.

2.3 Determination of antibody titer

2.3.1 Antibody titer against B. anthracis

2.3.1.1 Spore antigen preparation

The sporulated growth of virulent immunogenic strain on Difco tryptose agar were harvested in sterile saline.

The spores were recovered by centrifugation and washed 3 or more times by centrifugation from saline. The spore suspensions were made up to an optical density of 0.6 as measure in a spectophoto--meter at 640 nm.

2.3.1.2 Agglutination tests

Two-fold serial dilutions of the antiserum were mixed with equal volume (0.1 ml) of the spore suspension. The mixture were shaken vigorously for 30 to 60 minutes at room temperature. To each tube 0.8 ml of saline solution was then added, the agglutination reaction being read immidiately after shaking and again after over night storage at $4^{o}C$.

2.3.2 Antibody titer against P. multocida

2.3.2.1 Antigen preparation

The antigen consisted of organisms which was grown on Difco tryptose agar, incubated for 24 hr at $37^{o}C$ and washed off the agar with sterile normal saline to give a suspension containing about 1×10^{9} organisms per ml.

2.3.2.2 Agglutination tests

To 0.5 ml of two-fold serially diluted antiserum, equal volume of antigen were added, and tubes were incubated at $37^{o}C$ for 2 hr. Antiserum titers were recorded as the last tube showing complete agglutination and again after over night storage in the refrigerator.

2.4 Immunoglobulin preparation[94,95]

Each group of pooled sera (2.2.2) was concentrated and partially purified by ammonium sulfate precipitation in order to obtain immunoglobulins for passive protection tests.

The serum was precipitated by adding saturated ammonium sulfate solution to make a final concentration of 50% (v/v). The precipitate was collected by centrifugation and resuspended in the same volume of 0.01 M phosphate buffer saline (PBS) as the original serum. The process of precipitate was repeated (until the precipitate was cleared of hemoglobin) and the final precipitate was dissolved in the PBS at the half volume of the original serum. This was dialyzed against PBS at $4^{o}C$ and changed the PBS twice daily until it was tested to free from sulfate.

Estimation of the protein content in each group of immuno--globulin obtained by the method described by Lowry et al[96] and diluted the immunoglobulin solutions in each group to contain protein 25.0 mg / ml. The antibody titers of immunoglobulins were retitrated by method as described in 2.3.

2.5 Determination of the 50% Lethal Dose (LD_{50}) in mice

2.5.1 LD_{50} of B. anthracis

The spore suspension of a virulent strain was simply prepared as the method described for Bacillus anthracis vaccine preparation and stored in the refrigerator.

Mice were inoculated intraperitoneally with 0.2 ml

of ten-fold serial dilutions of the spore suspension. Ten mice were used for each dilution. The animals were observed for 14 days.

2.5.2 LD_{50} of *P. multocida*

The bacterial suspension of *P. multocida* was freshly prepared after 24 hr at 37°C incubation on blood tryptose agar.

Mice were inoculated intraperitoneally with 0.4 ml of two-fold serial dilutions of the bacterial suspension. Ten mice were also used for each dilution and mortality was observed for 3 days.

LD_{50} of *B. anthracis* as well as *P. multocida* was calculated by the method of Litchfield *et al*.[97]

2.6 Determination of the protective dose in mice[89]

The dose-volume of each injection per mouse was not less than 0.2 ml. The original immunoglobulin was diluted to the dose-volume with PBS.

2.6.1 Protective dose to *B. anthracis*

Immunoglobulin (25 mg / ml) obtained from immunization with monovalent vaccine of *B. anthracis* (anthrax spore vaccine) was administered subcutaneously in dose of 10, 5 and 2.5 mg in 0.4, 0.2 and 0.2 ml volume respectively to each group of 10 mice. After 24 hr, together with control group of 10 mice, they were challenged by the intraperitoneous injection of about 75 LD_{50} of

the virulent spore suspension of B. anthracis. Deaths and survivals were recorded for 14 days.

Both of immunoglobulins obtained from immunized individually with two types of bivalent vaccine in 2.1.2 were performed and processed in the same manner as above.

2.6.2 Protective dose to P. multocida

Immunoglobulin (25 mg / ml) obtained from immunization with monovalent vaccine of P. multocida (hemorrhagic septicemia vaccine) was administered subcutaneously in dose of 1.25, 0.625, 0.312, 0.156,and 0.078 mg in 0.2 ml volume to each group of 10 mice. After 24 hr, together with control group of 10 mice, they were challenged by the intraperitoneous injection of about 100 LD_{50} of the freshly homologous strain culture of P. multocida. Mortality were recorded for 3 days.

Both of immunoglobulins obtained from immunized individually with two types of bivalent vaccine in 2.1.2 were performed and processed in the same manner as above.

2.7 Mouse passive protection tests (89)

2.7.1 Passive protection test of B. anthracis

2.7.1.1 Single dose of immunoglobulin

Each group of 20 mice was administered subcutaneously by selected dose (10 mg / mouse after 2.6.1) of each immunoglobulin and challenged intraperitoneally with 75 LD_{50} of the spore suspension 24 hr later.

Control group of 20 mice was also exposed to the same challenged dose. Deaths and survivals were recorded for 14 days.

2.7.1.2 A booster dose of immunoglobulin

The selected dose (10 mg / mouse after 2.6.1) of each immunoglobulin was performed in the same manner as 2.7.1.1 to each group of mice. After the challenged dose, the another dose of each immunoglobulin was repeated by subcutaneous injection.

Control group of 20 mice was used. The animals were observed for 14 days.

2.7.2 Passive protection test of P. multocida

Each group of 20 mice was administered subcutaneously by selected dose (0.625 mg / mouse after 2.6.2) of each immunoglobulin and challenged intraperitoneally with 100 LD_{50} of the freshly prepared culture of P. multocida 24 hr later.

Control group of 20 mice was also exposed to the same challenged dose. Mortality was recorded for 3 days.