CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1 Organisms

1.1.1 Lyophilized P.multocida serotype 8:A, a local strain used for vaccine production of the Department of Livestock, was kindly obtained from the Division of Biological Products, the Department of Livestock Development, the Ministry of Agriculture and Cooperatives. This serotype was isolated from the outbreak of duck cholera in Thailand.

1.1.2 Lyophilized P.multocida, CU strain was kindly provided by Dr. B.W. Bierer, South Carolina Agricultural Experimental Station, Clemson University, Elgin, South Carolina, U.S.A.

Each lyophilized culture was reconstituted in a small volumn of broth and streaked on blood agar plates. After 18 hr of incubation at 37 °c, growth was harvested and suspended in sterile skimmed milk, and the suspension was divided into several 1 ml. vials which were frozen and maintained at -70 °c as stock cultures of each experiments.

1.2 Experimental Animals

About 1000 of 1-day old Khaki Campbell ducks (only male) purchased from a private company were fed with free-antibiotic food at the Veterinarian Student Training Center , Faculty of Veterinary Sciences, Chulalongkorn University, Nakornpratom. The house was cleaned and disinfected before starting the experiment.

1.3 Media

1.3.1 Brain-Heart-Infusion Broth (Difco Laboratory, U.S.A.)

1.3.2 Bacto Tryptose Broth with Thiamine (Difco Laboratory, U.S.A.)

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

1.3.4 Blood Agar

1.3.5 Flesh-Liver-Duck Media

1.3.6 Mac Conkey Agar (Difco Laboratory, U.S.A.)

1.3.7 Nitrate Broth (Difco Laboratory, U.S.A.)

1.4 Chemical

1.4.1 Bovine serum albumin (Sigma, U.S.A.)

1.4.2 Calcium chloride (May & Baker, England)

1.4.3 Formaldehyde solution 37-40 % wt/vol (BDH,England)

1.4.4 Hydrogen peroxide 3% solution

1.4.5 Magnesium chloride (May & Baker, England)

1.4.6 Magnesium sulfate hepta. hydrat (May & Baker, England)

1.4.7 Dimethyl- -napthylamine

1.4.8 Tetramethyl-p-phenylenediamine

1.4.9 Potassium chloride hexa. hydrate (May & Baker, England)

1.4.10 Potassium dihydrogen phosphate (May & Baker, England)

1.4.11 Sodium azide (BDH, England)

1.4.12 Sodium carbonate (Merck, Germany)

1.4.13 Sodium chloride (Merck, Germany)

1.4.14 Disodium hydrogen phosphate (Merck, Germany)

1.4.15 Sodium hydroxide (Merck, Germany)

1.4.16 Sodium potassium tartrate (Merck, Germany)

1.4.17 Sulfanilic acid

1.4.18 Tannic acid (Merck, Germany)

1.4.19 Zinc dust

1.5 Glassware and Others

1.5.1 Beakers (Pyrex, U.S.A)

1.5.2 Centrifuge tubes (Pyrex, U.S.A)

1.5.3 Disposable needles (Pyrex, U.S.A)

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

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

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

1.5.7 Measuring pipettes (Pyrex, U.S.A)

1.5.8 Pasteur pipettes (Pyrex, U.S.A)

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

1.5.10 Plastic tips

1.5.11 Screw-cap test tubes (Pyrex, U.S.A)

1.5.12 Serological test tubes (Pyrex, U.S.A)

1.5.13 Stirring rods

1.5.14 Stainless racks

1.5.15 Syringes (Surgical Manufacturing Co., Ltd)

1.5.16 V-microtiter plates (NUNC, Denmark)

1.6 Instruments

1.6.1 Analytical balance (Mettler, U.S.A)

1.6.2 Autoclave model H.A. 3D (Hirayama, Japan)

1.6.3 Bench-top centrifuge model Mistral 3000
(MSE Scientific Instruments, England)

1.6.4 Colony counter (New Branswich, U.S.A)

1.6.5 Deep freeze refrigerator (Forma-Bio-Freezer)

1.6.6 Incubator (Memmest)

1.6.7 Light microscope (Olympus)

1.6.8 pH meter (Beckmann, U.S.A)

1.6.9 Refrigerator (Philco)

1.6.10 Spectrophotometer model spectronic 710 (Baush Lomb, U.S.A)

1.6.11 Sonicator model UR-20P (TOMY-SEIXO, Japan)

1.6.12 Vertex cyclomixer (Vertex-genic, U.S.A)

1.6.13 Water-bath (Julabo, West Germany)

2. Methods

Experiment I : Growth Curves of P.multocida, CU Strain

The stock culture of P.multocida, CU strain at -70° c was streaked on blood agar plates and incubated at 37°c for 18 hr then the agar culture was suspended in 0.85 % saline to yield suspension with an absorbance of 0.15-0.16 at 540 nm used as a seed suspension.

Each three media of BHI broth (Difco), tryptose broth with thiamine (Difco), and flesh-liver-duck media (see appendix) was inoculated with 1.0 ml of the seed suspension per 100 ml media to yield the starting bacteria about 10^6 cells/ml. Flasks were incubated statically at 37°c and growth curves were obtained by sampling each culture at 0, 3, 6, 3, 12, 18, 24 hr and determining the optical density at 540 nm These samples were then assayed for viable counts using poured plate technique in tryptose agar.

Flasks containing 200 ml of BHI broth were modulated with 2.0 ml of seed suspension and incubated in different conditions, those are static at 37°c, static at 41.5°c, shaking 200 rpm at 37° c and shaking 200 rpm at 41.5°c. Growth curves were obtained as above in 48 hr incubation .

Experiment II : Virulence of the CU Strain in Various Ages of Ducks

Culture The stock culture of the CU strain propagated overnight on blood agar plate 2-4 colonies were transferred into BHI broth and incubated for 6 hr at 37° c with agitation. The concentration of bacteria was estimated by measuring the optical density at 540 nm and confirmed by plate counting on blood agar. The stock cultures yielded approximately $1x10^9$ - $4.8x10^9$ viable cells/ml were diluted into ten-fold serial dilution to obtain about 10^8, 10^7, 10^6, 10^5 viable cell/ml. respectively.

Experimental Animal About 480 ducks were used in this experiment. At the age of 1, 2, 3 and 4 wk each of 120 ducks was selected randomly. For each time, 20 ducks,were housed, in each of 6 isolated units.

Experimental Design Ducks in the first unit were inoculated subcutaneously with 1.0 ml of the stock culture. Those in the second unit recieved 1: 10 dilution; in the third, 1: 100 dilution; in the fourth unit, 1: 1,000 dilution ; in the fifth , 1: 10,000 dilution and in the sixth unit were served as control group. Mortalities were recorded daily for 14 days after inoculation. All ducks that died were necropsied and their livers were cultured for P.multocida by streaking on blood agar plates. Identification of the P.multocida was based on the results from gram staining, colony morphology oxidase test, catalase test, nitrate reduction test and growth on Mac Conkey agar.

Experiment III : Protective Immunity and Antibody Responses

Vaccine Preparation The stock culture of the CU strain was streaked on blood agar and incubated for 18 hr at 37°c. The agar cultures were suspended in 0.85 % saline and adjusted to obtain optical density about 0.15-0.16 at 540 nm. BHI broth was inoculated with 1.0 ml of this seed suspension per 100 ml of broth and incubated at 37° c without agitation for 6-9 hr The broth cultures were sampled aseptically to check for purity by gram staining and colonial morphology forming on blood agar. During the time of vaccination, such culture was determined the number of colony-forming units per ml (CFU/ml) by plate counting on blood agar. The vaccines contained about $1x10^9$, $4.4x10^8$, $2.0x10^8$ CFU/ml .

Experimental Animal (Four hundreds of 6 - wk - old ducks) were placed in 4 seperated wire pen (about 100 ducks per pen). Each group was identified by metal clips with group number on their legs.

Serum Collection Ducks were bled 1-4 ml from wing vein before each challenge and prior to the first vaccination. Blood was allowed to clot for several hours at room temperature. Then it was centrifuged and serum was collected, labeled and stored at -20°c for determining antibody titers.

Challenge The 8:A strain culture (-70°c) was streaked on blood agar and incubated at 37°c overnight then 2-4 colonies were grown further in BHI broth for 6-8 hr The broth cultures were diluted to approximately 10^7 CFU/ml (the exact dose challenge for each time as shown in Fig.1). Each duck was challenged 1.0 ml of this suspension intramuscularly. The mortalities were observed up to a period of 7 days.

Vaccination Programme and Sample Collection (... Fig 1)

Subcutanous vaccinated group one hundred ducks were inoculated with 1.Ø ml of the CU live vaccine subcutanously (S/C) in the mid portion of the neck. This vaccine contained approximately $1x10^{9}$ viable cells per ml Afterward, these ducks were further divided into 3 subgroups (Number of ducks was 4Ø, 4Ø, 2Ø respectively).

Sub group 1 (Single vaccination) At 1, 2, 4 and 8 wk postvaccination, not less than 5 ducks of each time were randomly selected, bled and challenged with the 8: A strain of P.multocida.

Sub group 2 (Double vaccination of 1 month interval) About 4Ø ducks were given the second vaccination (1 ml, S/C) 1 month after the first vaccination. The vaccine contained about $4.4x10^{8}$ viable bacteria/ml At 1, 2, 4 and 8 wk after the second vaccination, some of ducks were sampled for bleeding and challenging.

Sub group 3 (Double vaccination of 2 month interval) About 2Ø ducks were given second vaccination (1 ml, S/C) 2 months after the first vaccination. This vaccine contained about $3.7x10^{8}$ viable cells /ml Then at 1, 2 and 4 wk after the second vaccination, all ducks were bled and were challenged only at the last time.

Oral vaccinated group one hundred ducks were vaccinated orally through a modified oral tube (small plastic tube fixed onto the tip of a 5 ml syringe). By this way each duck recieved 1 ml of vaccine exactly. Afterwards, the ducks were further subdivided into 3 subgroups (single vaccination, double vaccination of 1 month interval and double vaccination of 2 month interval). Scheme of

sampling was the same as the S/C vaccination group.

Drinking water vaccinated group one hundred ducks were vaccinated by drinking water accomplished by mixing 100 ml of fresh vaccine in drinking water at a ratio of 1:20 and administering this until all was consumed. Thus the 3 hr deprived ducks would recieved 1 ml of fresh vaccine per average one duck. Afterwards the duck were further subdivided into 3 subgroups and the procedure was followed as in the S/C vaccination group.

Unvaccinated control one hundred ducks were served as unvaccinated challenged control.

Evaluating of Fowl Cholera Vaccine (101)

% protection = % survival of vaccinated group - % survival of unvaccinated group (on each challenge)

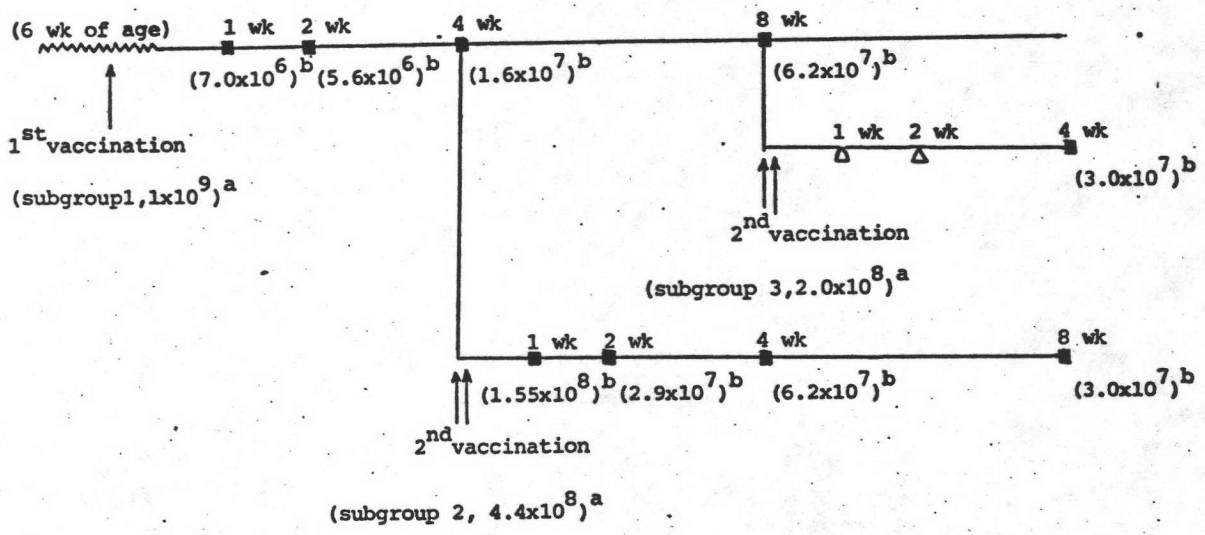

Fig 1 : Vaccination programme and sample collection

■ = bleeding and challenge

Δ = bleeding

wk = weeks after each vaccination

a = dose of vaccination (viable bacteria per duck)

b = dose of challenge (viable bacteria per duck)

Determination of Antibody Titers

1. Antigen preparation : Antigens of both 8:A and CU strain were prepared separately by 3 methods.

1. Autoclaved antigen for tube agglutination (TA) test
2. Sonicated cell antigen for passive hemagglutination (PHA)test
3. Capsule antigen for passive hemagglutination (PHA)test

Stock culture (-70 °c) must be incubated on blood agar at 37 °c for 18 hr Then the agar cultures were grown further in BHI broth at 37 °c by shaking at repeatly 200 rpm for 18 hr . The broth culture was harevested and washed 4 times by washing with phosphate buffer saline (PBS) pH 7.2 and centrifugation at 1,200 g, 4 °c for 30 minutes . Packed cell of each strain were prepared for 3 antigens.

1.1 Autoclaved antigen (102) : A part of packed cells was suspended in 0.85 % saline and autoclaved 121 °c for 15 minutes. The suspension was diluted to optimal density (O.D.) ofabout 0.6 at 520 nm.

1.2 Sonicated antigen (103, 104) : Suspension of washed cells in a little of PBS pH 7.2 was soicately disrupted with a handy sonicator (UR-20 p, Tomy seiko, JAPAN) at 70 % of maximum power to accomplish 70-80 % broken cells. (checked by gram staining)

1.3 Capsular antigen (40, 57) Packed cells 1.8 gm was suspended in 2.5 % saline and heated with shaking at 56 °c for 1 hr then centrifuged at 20,000 g, 4 °c for 35 minutes. The supernatant was dialysed with 0.85 % saline at 4 °c for 24 hr .

2. Determination of Antibody Titer

2.1 Tube Agglutination Test (102, 105)

Two-fold serial dilution of the serum were carried out in test tubes and mixed with an equal volume (0.2 ml) of the autoclaved antigen. The mixture was shaken, and incubated for 12 hr at 37 °c. Antisera titers were recorded as the last tube showing complete agglutination and the titers were observed again, after overnight storage at 4 °c.

2.2 Passive Hemagglutination Test (PHA) (106)

2.2.1 Preparation of 2 % tannin-formalinized treated SRBC

A 100 ml suspension of fresh sheep red blood cells (SRBC) in Alsever's solution was kept at 4 °c for 3-5 days before use. The SRBC were washed four times with normal saline soluion (NSS). There should be no hemolysis. Eight millitres of packed SRBC were resupended in 72 ml of NSS. Then 80 ml of 7.5 % formalin-saline solution were added gradually into SRBC suspension. The mixture was incubated for 18-20 hr at 37 ° c and gently agitated. After incubation, the mixture was washed 4 times with NSS and made into a 10 % formaliniged cells in NSS. This suspension was stored at 4 °c and could be used for at least 1-2 years.

Sixteen millitres of 10 % formalinized SRBC were washed with NSS oncebefore use. Then the pack cells were mixed with 80 ml of phosphate buffer saline solution (PBS) pH 7.2 in a 250 ml flask to make a 2 % suspension. This was mixed vigorously with an equal volume of 1:40,000 tannic acid in NSS and incubated for 30 minutes in waterbath at 37 °c. The mixture was washed twice with PBS and

made into 2% tannin-formalinized SRBC by resuspending it in 80 ml of PBS. These cells could be stored at 4 °c for at least 6 months.

2.2.2 Sensitization of tannin-formalinized SRBC

The optimal amount of antigen for sensitization was determined by titrating a series of diluted antigen with equal volumn of 2 % tannin-formilinized cell. The mixture was incubated for 1 hr 37 °c in water bath then the cells were washed twice with PBS and resuspended into 0.25 % bovine serum albumin in PBS (BSA-PBS) to yield a 1 % cell suspension. The sensitized cells were tested with positive serum in PHA procedure (2.23) to determine the optimal dilution of antigen that gives highest titer with positive serum.

2.2.3 Passive hemagglutination procedure

Sera were heat-inactivated at 56 °c for 30 minutes and absorbed with an equal volume of 10% formalinized cells at 37 °c for 2 hr to remove heterophile antibody. Serial two-fold dilutions of specimens tested were prepared in a 25 µl volume (0.25% BSA-PBS) with microtiter diluter in v-shaped plastic microtration plates. Twenty-five miccrolitres of 1% sensitized cell suspension were added to each well. The plates were shaken and incubated at room temperature. The hemagglutination patterns were read after 1-2 hr incubation.

For each run, sensitized cells plus diluent and unsensitized cell plus tested serum were performed as controls.