Chapter 3

Materials and Methods

1. Materials

1.1 Organism : Lyophilized *Pasteurella multocida* serotype 8:A was 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 fowl cholera in ducks in Thailand. The Department of Livestock Development used this strain for the production of vaccine.

1.2 Experimental animal : Duck : Four hundred and fifty-three kaki-chembell ducks were used . One-day-old ducklings were purchased and raised in the Veterinarian Student Training Center in the Faculty of Veterinary Science at Nakornpratom. The ducks which were 10 weeks old at the time of vaccination were housed together.

1.3 Media

1.3.1 Bacto Tryptose Broth with Thaimine (Difco Laboratory, U.S.A.)

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

1.3.3 Blood Agar

1.4 Chemical

1.4.1 Albumin bovine (Sigma, U.S.A)

1.4.2 Aluminium hydroxide gel
(T.S. polyproduct, Thailand)

1.4.3 Arlacel C, Sorbitan sesquioleate

1.4.4 Benzyl alcohol (Mallinckrodt, U.S.A)

1.4.5 Corn oil (Mazola, U.S.A.)

1.4.6 Disodium hydrogen phosphate
(May & Baker, England)

1.4.7 Formaldehyde solution 35 Gew%
(Merck, Germany)

1.4.8 Glutaraldehyde (Sigma, U.S.A.)

1.4.9 Hydrochloric acid (Merck, Germany)

1.4.10 Mineral oil

1.4.11 Sodium azide (BDH, England)

1.4.12 Sodium chloride (May & Baker, England)

1.4.13 Sodium dihydrogen phosphate
(May & Baker, England)

1.4.14 Span 80, Sorbitan mono-oleate
(Sigma, U.S.A.)

1.4.15 Tannic acid

1.4.16 Tween 80, Polyoxyethylene (20) sorbitan mono-oleate (BDH, England)

1.5 <u>Glasswares</u>

1.5.1 Beakers

1.5.2 Erlenmeyer flasks

1.5.3 Funnels

1.5.4 Glass beads

1.5.5 Glass slides

1.5.6 Measuring cylinders

1.5.7 Measuring pipettes

1.5.8 Pasteur pipettes

1.5.9 Petri dish

1.5.10 Roux bottle

1.5.11 Serological test tube

1.5.12 Stirring rod

1.5.13 Syringe

1.5.14 Test tube with screw cap

1.6 Instruments

1.6.1 Autoclave model HA-3D (Hirayama, Japan)

1.6.2 Bench-Top centrifuge model centra-4 (IEC, U.S.A.)

1.6.3 Colony counter (New Branswick, U.S.A.)

1.6.4 Deep freeze refrigerator

1.6.5 Hot plate

1.6.6 Homogenizer (Ystral, Japan)

1.6.7 Light microscope

1.6.8 Larminar flow

1.6.9 pH meter (Beckman, U.S.A.)

1.6.10 Refrigerated centrifuge (Hitachi, Japan)

1.6.11 Refrigerator (Hitachi, Japan)

1.6.12 Spectrophotometer model Spectronic 710 (Bausch & Lomb, U.S.A.)

1.6.13 Sonicator, Heat system ultrasonic Inc Model W-375

1.6.14 Vertex cyclomixer (Vortex-genic, U.S.A.)

1.6.15 Water bath (Julabo, West Germany)

1.7 <u>Others</u>

1.7.1 Disposable needle

1.7.2 Filter paper no.1 Qualitative (Whatman, England)

1.7.3 U-bottom Microtitration plate (Nunc, Denmark)

1.7.4 V-bottom Microtitration plate (Nunc, Denmark)

2. Methods

2.1 Preparation of four types of vaccine

2.1.1 Formalin-killed whole-cell vaccine (FDC)

The preparation of formalin-killed whole - cell vaccine was modified from the method of Layton (3), Heddleston and Hall (7).

The seed culture for vaccines was prepared by reconstituting the lyophilized stock culture of Pasteurella multocida serotype 8:A in a medium containing of 0.5 ml of Tryptose broth. This suspension was streaked on blood agar plates and incubated for 20-24 hours at 37° C. Colonies of P.multocida were selected from the blood ager and were used to inoculate two 15 ml tubes of trytose broth. The broth culture was incubated for 6 hours at 37°C. After incubation, asample was taken aseptically from the culture. The sample was diluted and plated on blood agar to check for purity and to determine the number of colony-forming units (CFU) per ml. The seed culture, containing about 1.48×10^{9} CFU per ml., was divided into 2 parts. One was kept at -70°C. as a stock culture and used in the protection study. The other was used to prepare Formalin-killed whole-cell vaccine on the same day.

To prepare the formalin-killed whole

cell vaccine, 10 ml of the seed culture was transferred to 2,000 ml of tryptose broth. The broth culture was incubated for 20-24 hours at 37°C and continuously shaken at 200 oscillations per minute. After incubation, a sample was taken, aseptically, from the broth culture. Part of this sample was diluted and plated on blood agar and Tryptose agar to check for purity and to determine the number fo colony-forming units per ml. The concentration of this vaccine was 7.7×10^7 CFU per ml. The rest of the sample was transferred to a cuvette to determine absorbance at 525 nm. To adjust the absorbance to 1.624 which was the absorbance of vaccine from The Department of Livestock Development, the broth culture was placed in a centrifuge and then the supernatant drained away through a pipette.

Formalin was then added to the broth culture to make a final concentration of 0.25 % of the total volume and kept in a refrigerator overnight. A sample of the formalin-killed whole cell vaccine or bacterin was tested by plating it on Tryptose agar to determine its sterility before use. This formalin-killed whole - cell vaccine was used to prepare three other types of vaccine.

2.1.2 <u>Mineral - oil - adjuvanted vaccine</u> (MDC) (Modified from the method of Heddleston and Reisinger) (4).

Mineral oil, Benzyl alcohol and Span 80 were heated together in a 250 ml. Beaker at 37 °C while the

formalin-killed whole-cell and Tween 80 were heated together in another Beaker at the same temperature. Then the second part was added to the first part and mixed together with a homogenizer (speed 1) for 1 minute, then gradually turned to speed 3 for 2 minutes. This emulsified vaccine was of the oil-in-water type. The mixture contained :

Mineral oil	44.15	g.
Span80	4.11	g.
Tween80	1.39	g.
Benzyl alcohol	0.35	g.
Bacterin	50.00	g.
(Formalin-killed whole-cell vaccine)		

2.1.3 Corn-oil-adjuvanted Vaccine (CDC)

To prepare corn oil adjuvanted vaccine, corn oil and Aracel C were heated together in a 250 ml. Beaker at 35°C while bacterin from the formalin-killed whole cell vaccine and Tween 80 were heated in another Beaker at 35°C. The latter part was then added to the first and mixed together with a Homogenizer at speed 1 for 1 minute and immedately turned to speed 3 for minutes. This vaccine was in the form of water-in-oil emulsion. The following mixture was emulsified.

Corn oil	38.50 g.
Aracel C	8.76 g.
Tween80	2.74 g.
Bacterin	50.00 g.

2.1.4 Aluminum hydroxide-absorbed Vaccine (ADC) (10)

One part aluminium hydroxide gel was mixed with six parts bacterin in a homogenizer at speed 1 and gradually turned to speed 3 for 2 minutes. The following mixture was susperded

Aluminium hydroxide gel	10.00 g.
Bacterin	60.00 g.

2.2 Livestock Department Vaccine (DCL)

Livestock Department Vaccine is formalin-killed whole cell vaccine obtained from the Department of Livestock Development. The absorbance was 1.624 at 525 nm. This type of vaccine was used to compare with the various preparations of vaccines in this study.

2.3 Preparation of Antigens

2.3.1 Agglutinating antigen

To prepare antigen for the detection of agglutinating antibody titer, P.multocida serotype 8:A on

blood agar was inoculated into tubes of 15 ml. Tryptose broth. After incubation for 6 hours at 37 °C, 5 ml of the broth culture was used to inoculate into three flasks, each containing 1,000 ml of Tryptose broth. These flasks were incubated for 20-24 hours at 37°C and continuously shaken at 200 oscillations per minute. The bacteria were then centrifuged at 4,500 g for 30 minutes and washed twice with sterile saline solution. The pellet was pooled to yield a suspension which was later divided into three parts for the preparation of autoclaved antigen, formalinized antigen and sonicated antigen.

2.3.1.1 Autoclaved Antigen (Modified from the method of Alexander and Soltys) (63)

One part of the suspension was autoclaved at 121°C for 15 min

2.3.1.2 Formalinized Antigen (4,7)

Formalin was added to give a final concentration of 0.25 percent.

2.3.1.3 HCl-Treated Antigen (64)

P.multocida 8:A was grown on tryptose agar in Roux bottles for 18 hours at 37°C. The bacteria were washed from the agar surfaces with sterile

saline solution and pooled in a sterile flask. The concentration of P.multocida was adjusted to 10^9 cells/ml of saline solution. Formalin was then added to the flask to give a final concentration of 0.25 % of the total volume. The flask was kept at room temperature for 18 hours. The formalinized bacteria were centrifuged at 21,000g for 15 minutes. The pellet was then suspended in 150 ml of 1N HCl saline solution and incubated at 37° C for 18 hours. These cells were centrifuged again and washed twice with formalinized phosphate buffered saline solution (pH 5.8). The cell concentration was then adjusted to give a value of 0.6 optical density on a spectrophotometer at a wavelength of 525 nm.

2.3.1.4 Living cell Antigen

P.multocida was grown on Tryptose agar in Roux bottles for 18 hours at 37° C. The bacteria were washed from the agar surfaces with sterile saline solution and pooled in a sterile flask. The antigen was adjusted to an absorbance of 0.6 at 525 nm. and used on the same day.

Each antigen was kept in a refrigerator. These antigens were adjusted to yield an optimal density of 0.6 at 525 nm before use.

2.3.2 Indirect hemagglutinating antigen(75)

One part of the suspension was sonically disrupted with a sonicator, operated at level 5 of its

power, for 10 minutes to make sonicated antigen. The cells were pellet-centrifuged at 10,000 g for 20 minutes and the supernatant fluid was designated sonicated antigen or indirect hemagglutinating antigen. The antigen was preserved with 0.1% sodium azide and kept at 4°C.The supernatant fluid was determined for protein content by the method described by Lowery et al.(77). The protein content of this sonicated antigen was 1.665 mg. per ml. This sonicated antigen was diluted 1:45 with sterile phospate buffer saline solution (PBS) before use.

2.4 Determination of antibody titer

2.4.1 Agglutination test

2.4.1.1 Tube agglutination method (63,64,78)

Two-fold serial dilutions 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, after which the tubes were incubated for 12 hours at 37°C. Antisera titers were recorded as the last tube showed complete agglutination and again, after overnight storage in the refrigerator at 5°C. Antigens, prepared with different methods, were used to detect antibody titers with serum samples obtained from ducks vaccinated with MDC and CDC vaccine.

2.4.1.2 Microtitration method

U-shaped microtitration plates were used. Two-fold dilutions of the serum were mixed with an equal volume (50 ul) of the autoclaved antigens. The mixture was shaken, after which the plates were then incubated for 18 hr at 37°C.Antiserum titers were recorded as the last well showing complete agglutination.

The tube agglutination Method and microtitration method were determined concomitantly. Serum samples from the Livestock Department group were used.

2.4.2 Indirect hemagglutination test.(IHA) (79)

2.4.2.1 Preparation of 2 % tannin-formamide treated sheep erythrocytes

A 100 ml suspension of fresh sheep red blood cell (SRBC) in Alsever's solution was kept in refrigerator for 3-5 days before use.The SRBC were washed four times with sterile saline solution. 8ml. of packed sheep red blood cell (PSRBC) were resuspended in 72 ml.of 0.9 % normal saline solution. Then 80ml.of 7.5 % formalin-saline solution were added to the SRBC suspension. The mixture was incubated for 18-20 hr at 37°C and occasionally shaken. After incubation, the mixture was washed 4 times with 0.9 % sterile saline solution and made into a 10 % suspension (10 % formamide treated sheep red blood cell). This suspension was stored at 4°C and could be used for at least 1-2 years.

The suspension of 10 % formamide-

treated SRBC was washed with 0.9 % sterile saline solution once before use. 1.6 ml of PRBC 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 together in a flask with 1:40,000 tannic acid in a 0.9 % saline solution equal to the cell suspension and incubated for 30 minutes at 37°C. The mixture was washed twice with PBS, pH 7.2 and made into 2 % tannin-formamide-treated sheep erythrocytes by resuspending it in 80 ml of PBS. This treated SRBC stored under refrigeration could be used for at least 6 months.

2.4.2.2 Sensitization of tannin-formamide-treated sheep erythrocytes

The optimal dilution of antigen for the sensitization of tannin-formamide-treated SRBC was determined.The dilution of antigen used in the IHA test was 1:45.

A 2 % suspension of tannin formamide-treated sheep erythrocytes was mixed with an equal volume of 1:45 dilution of sonicated antigen. The mixture was incubated for 1 hour at 37°C. The sensitized cells were washed twice with PBS by centrifugation and suspended in PBS containing 0.25 % bovine serum albumin (BSA-PBS) to yield a 1 % suspension.

2.4.2.3 IHA test

The antisera must be absorbed by the addition of an equal volume of 2 % formamide-treated sheep erythrocytes before use. After incubation for 2 hours at 37°C, the cells were removed by centrifugation.

The IHA test was performed with a microtiter system. Two fold serial dilutions of antiserum (ranging from 1:2-1:4096) were made in BSA-PBS, and 25 ul. of the sensitized SRBC were added to 25 ul. of the diluted antiserum in V-bottom microtitration plates. The plates were shaken and allowed to stand for 1 hour at 25°C. The IHA titer was expressed as the reciprocal of the highest dilution of the serum and showed a definite, positive pattern when compared with the pattern of the negative control. Controls consisted of unsensitized SRBC plus test serum and sensitized SRBC plus diluent.

2.5 Vaccination: experimental design

Four hundred and fifty-three ducks were identified by numbers on their leg bands and divided into six groups. Groups I, II, III, IV, and V, vaccinated with FDC, MDC, CDC, ADC, and DCL vaccine respectively, and were confined together in a pen. Group VI which was not vaccinated served as a control group. All the ducks whether vaccinated or not were allowed to mingle within the same pen.

Prior to vaccination, 5 ml of blood was taken from the wing veins of 139 ducks. The serum obtained in this manner was stored at -20°C for later testing. All the serum samples were tested for agglutinating antibodies.

Group I Ducks vaccinated with formalin-killed whole-cell vaccine.

Group I, all females, was divided into 2 subgroups. Subgroup I, consisting of 32 ducks, was given one dose of 2 ml intramuscular injection of formalin-killed whole- cell vaccine. Subgroup II, consisting of 31 ducks, received two doses of 2 ml of the vaccine intramuscularly, 30 days internally. Both subgroups received their first vaccination at the same time.

At 15,30,60, and 90 days postvaccination, 19,14,9, and 4 ducks, respectively from subgroup I were selected at random for bleeding. After bleeding, 7,8,,7, and 4 ducks were challenged with P.multocida 8:A. All ducks were observed for 7 days. From the ducks that died cultures were prepared to confirm a diagnosis of fowl cholera. All the survivors were bled to determine for antibody titer.

At 15,30,60,90,120, and 135 days after the second vaccination 14,15,11,11,4,and 4 ducks from subgroup II were selected for bleeding.The immunity of each vaccinated duck

was observed by challenged intramascularly at 30,90, and 135 days after the second vaccination using 5,6, and 5 ducks respectively. The observation were carried out in a like manner to those in subgroup I.

All of the serum samples were determined by tube agglutination (TA) and indirect hemagglutination (IHA) test.

Each duck in subgroup I received approximately $2x10^4$ CFU/ml as a challenge dose while subgroup II received approximately $2x10^5$ CFU/ml. The challenge dose of $1.6x10^9$ CFU/ml, was administered 135 days after the second vaccination.

A virulent culture of P.multocida serotype 8:A was used to challenge the ducks' immunity. The culture were kept at -70°C until needed for challenge, and then was inoculated on blood agar, incubated for 20 hours at 37°C. Colonies of P.multocida were selected from plates and transferred to 10-15 ml of Tryptose broth. After 6 hours'incubation at 37°C,a sample of the broth culture was diluted and plated on blood agar to determine the CFU per ml. The concentration of the culture was then adjusted to the following level: $2x10^4$, $2x10^5$, and $1.6x10^9$ CFU/ml.

Group II Ducks vaccinated with mineral-oil-adjuvanted vaccine

Group II, comprising 36 males and 54 females,

was divided into 2 subgroups. Subgroup I, consisting of 40 ducks, was given one dose of 2 ml of mineral-oil-adjuvanted vaccine. The vaccine was administered subcutaneously into the midportion of the neck. Then 30 days later the second subgroup consisting of 50 ducks, receieved two doses of 2 ml of the vaccine. Both subgroups received their first vaccination at the same time.

At the same time as the ducks from group I (ducks vaccinated with formalin killed whole-cell vaccine), 39,17,14, and 2 ducks, respectively were selected for bleeding.

Like group I, 11,9,9,and 3 ducks,respectively, were selected and removed so that they could be given a challenge dose.

The second dose was administered 30 days postvaccination in subgroup II. The same procedure as group I was taken, 12,20,10,7,2, and 3 ducks respectively were selected for bleeding. At 30,90, and 135 days after the second vaccination, 8,6, and 4 ducks respectively were removed for challenge.

All procedure for clallenge and detecting antibody level were performed as above.

<u>Group III Ducks vaccinated with corn- oil-adjuvanted vaccine</u>

Group III, comprising 36 males and 54 females, was divided into 2 subgroups, Subgroup I, consisting of 40 ducks, was given one dose of 2ml of corn -oil- adjuvanted vaccine. Subgroup II, consisting of 50 ducks, received two dose of 2ml of the vaccine. The vaccine was administered subcutaneously into the midportion of the neck. The administration of both single and double doses of the vaccine was of the same duration as with group I.

Like group I, at 15,30,60, and 90 days postvaccination, 34,15,15, and 4 ducks respectively were selected for bleeding. At the same time, 10,10,10, and 4 ducks respectively were selected and removed for challenge.

The second dose of 2ml of the vaccine was given in subgroup II, 30 days postvaccination. Just as with group I ,11,19,11,9,4,and 6 ducks respectively were selected for bleeding. At 30,90, and 135 day after the vaccination, 9,6, and 5 ducks respectively were selected and removed for challenge.

Group IV Ducks vaccinated with aluminium-hydroxide-absorbed vaccine

Group IV, comprised entirely of females, was divided into 2 subgroups. Subgroup I,consisting of 40 ducks,

was given one dose of 2ml of aluminium hydroxide-absorbed vaccine. The 50 ducks in subgroup II were given two doses each of 2 ml of the vaccine. The first and second vaccination was administered similar to group I.

The vaccine was administered subcutanceously into the midportion of the neck. As with group I,at 15,30,60, and 90 days postvaccination, 28,13,12, and 2 ducks respectively were selected for bleeding while at the same time, 10,10,6, and 3 ducks respectively were removed for challenge.

The second dose was given 30 days postvaccination. Just as with group I, 12,9,10,20,8, and 10 ducks respectively were selected for bleeding and 8,10, and 10 ducks respectively was selected and removed for challenge at 30,90,135 days after the second vaccination.

<u>Group V Ducks vaccinated with Livestock Department vaccine</u>

The ducks in Group V, comprised entirely females, were divided into 2 subgroups. Subgroup I, consisting of 20 ducks, was given one dose of 2 ml of Livestock Department vaccine. Subgroup II, consisting of 30 ducks, was given two dose of 2 ml of the vaccine. The first and second vaccinations were conducted in a manner similar to group I.

The vaccine was administered intramuscularly. As with group I, at 15,30,60, and 90 days postvaccination, 20,10,5, and 2 ducks respectively were selected. At the same time, after bleeding, 6,6,3,and 2 ducks respectively were selected and removed for challenge.

The second vaccination was given in subgroup II at 30 days after the first vaccination.Just as with group I,13,13,9,6,4, and 5 ducks were selected for bleeding, after which that 5,6, and 4 respectively were selected and removed for challenge at 30, 90, and 135 days after the second vaccination.

Group VI: the control group

Group VI consisted of a control group of 70 unvaccinated ducks. At 15,30,60,and 90 days postvaccination 35, 16, 19, and 13 ducks respectively were selected for bleeding. At the same time, 10, 11,7, and 6 ducks were removed and challenged. They were exposed to the same challenge dose as the vaccinated ducks in subgroup I.

At 15,90,120,135 days after the second vaccination, 18,15,5, and 4 ducks respectively were selected for bleeding. At 30,90, and 135 days after the second vaccination, 5,4, and 7 ducks respectively were removed and chellenged. They' were exposed to the same

challenge dose as the vaccinated ducks in subgroup II.

<u>Evaluating a fowl cholera vaccine</u>

Potency = % survival of vaccinated duck - % survival of unvaccinated duck $\nless$ 60 (89).