## CHAPTER III

## MATERIALS AND METHODS

### Materials

1.1 Live attenuated vaccine (DP-L)

Chicken embryo-adapted attenuated vaccine was kindly supplied by the Department of Livestock Development, Ministry of Agriculture and Cooperatives.

1.2 Virulent duck plague virus

The livers of duck infected with duck plague virus were obtained from Dr. Urasri Tantaswadi of the Department of Livestock Development, Ministry of Agriculture and Cooperatives.

1.3 Special chemicals

2-Bromoethylamoniumbromide,the inactivator, was supplied by the Department of Livestock Development, Ministry of Agriculture and Cooperatives.

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1.4 Experimental animals

1-day-old Khaki Campbell ducklings (both male and female) purchased from a private company were fed at the Veterinarian Student Training Center, Faculty of Veterinary Sciences, Chulalongkorn University, Nakornpratom.

2 months later, 400 ducks were used in the experiment.

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1.5 Embryonating eggs

Leghorn chicken and Pekin duck embryonating eggs which were supplied by the Animal Health and Technical Service Operation, Charoen Pokapan Group of companies were used to prepare chicken and duck embryo fibroblast cells.

Methods

1. Vaccine Preparation

1.1 Live attenuated vaccine (DP-L)

Lyophilized vaccine was reconstituted with 200 ml diluent, after which it was ready for use at the recommended dose.(1 ml/dose)

For titration, the lyophilized vaccine was thawed with 1 ml of PBS (appendix II) and ten-fold dilutions prepared from the resuspended vaccine with PBS. 0.1 ml of each dilution was dropped into 96-well flat-bottomed microplates. An equal volume of freshly prepared chicken embryo fibroblast (CEF, appendix I) ($1 \times 10^6$ cell/ml) was added into each well after which the microplate was incubated at $37^{o}C$ in an atmosphere containing 5% $CO_2$. Five days later, the plates were examined to determine the cytopathic effect (CPE) which was then recorded. The titer was calculated by the method used by Reed and Muench (76) and expressed as tissue culture infectious dose 50% ($TCID_{50}$); base on the results, the viral content in 1 dose of this vaccine (1 ml) could consequently be calculated.

1.2 Inactivated vaccine without adjuvant (BEI-DP)

1.2.1 Preparation of specimen for inoculation into cell cultures.

Livers from duck that had died of duck plague was made into a 10% suspension in PBS. The suspension was clarified by centrifugation at 300 g for 20 minutes at 4 °C. The supernatant was decontaminated by pressure filtration through a 0.45 um membrane. The filtrate was then ready for inoculation.

1.2.2 Culture of duck plaque virus in duck embryo fibroblast (DEF)

Confluent layers of DEF were prepared in a Roux bottle (appendix 1). The cell sheet was then inoculated with the filtrate (prepared in 1.2.1) and adsorbtion was effected for 1 hour at 37 C. Cultures were then added with maintainance medium (appendix II) and incubated at 37 °C. After inoculation, the cultures were examined daily for CPE. Infected cells, showing about 80% CPE, were frozen at-70 °C and thawed immediately for 3 times. The viral suspension received a further two passages of DEF to increase its titer and virulence. Tests were performed to determine the final titer (after passing the virus 3 times through DEF) and the titer was expressed as $TCID_{50}$ and duck lethal dose50% ($DLD_{50}$)

1.2.3 Titrate duck plaque virus in cell cultures and experimental animals

- cell cultures

The same procedure was followed as with the titration of DP-L but DEF was used instead of CEF.

- experimental animals

The same procedure was followed as with the titration of DP-L but 2-month-old ducks were used as the host system. The dosage was 1 ml, and the route of inoculation was by means of intramuscular injection.

The ducks'mortality rate was recorded daily during the following 2 weeks. In each case, the cause of death was diagnosed by looking for the lesions that are peculiar to duck plague.

1.2.4 Inactivation of duck plague virus

2 Bromoethylamoniumbromide (BEI) was used in a final concentration of 0.05% to inactivate the duck plague virus. The temperature at which inactivation took place was 37 $^{o}$C; sodium thiosulfate was used to stop the reaction of the BEI.

- The time used to render the duck plague virus completely inactive (6)

Twelve vials, each containing 2 ml of viral suspension, were placed in a 37 $^{o}$C waterbath. Six vials were added with 5 ul of PBS containing 20% BEI (appendix II) The other six were added with 5 ul of PBS only. Each suspension was thoroughly mixed with vortex mixer. During the following 6 hours of incubation, the inactivated and control vials were taken out of the waterbath at 1 hourly intervals and the reaction of BEI stopped by mixing 1.8 ml of virus with 0.2 ml of 20% sodium thiosulfate (appendix II). Titration of the virus in each vial was followed. The titration method was the same as in 1.2.3.

1.2.5 Preparation of vaccine

Duck plague virus was inactivated with BEI for 6 hours by the method already described in 1.2.4 The vaccine was poured into a bottle and stored at 4 $^{\circ}$C.

In order to test the safety, 1 ml of the vaccine was administered intramuscularly to 2-month-old ducks. They were observed for the following 2 weeks to see if death would ensue or for signs of sickness.

Two lots of this vaccine were prepared. The first lot was safe enough to administer but the amount was not sufficient in case of revaccination, so the second lot of vaccine was prepared. The method of preparation was identical to that followed with the first lot, including the concentration of inactivator and the length of time for which it was exposed to it. After inactivation, the second vaccine was used promptly without checking for safety because the schedule for the second vaccination was very tight. Six days after the second vaccination, an outbreak of duck plague occurred.

1.3 Inactivated vaccine with adjuvant (BEI-DPM) (78)

Half the amount of inactivated duck plague virus prepared in 1.2 was mixed with mineral oil by using the following method and formulation. For the aqueous phase, 50 g of viral suspension was mixed with 1.39 g of tween 80 by using a stirring rod and incubated in a 37 $^{\circ}$C waterbath. For the oil phase, 44 g of mineral oil was mixed with 4.11 g of span 80 and incubated in the same manner as in the aqueous phase. The aqueous phase was then poured into the oil phase. The mixture was homogenized in a Virtis mixer vessel at 1/5

maximum speed for 20 minutes (at 5 minute intervals). The resulting vaccine, in emulsion form, was dispensed into a bottle and stored at 4 C.

2. Experimental Animal

400, 2 1/2-month-old, ducks that were housed together were used. Three groups (100 ducks per group) were vaccinated, while the last group was unvaccinated. Metal clips with numbers were used to identify the ducks. After the outbreak, unvaccinated ducks which had hitherto been kept seperate, were included to ensure the virulence of the duck plague virus in the protective immunity.

3. Vaccination Programme and Sample Collection

Prior to vaccination, 20-30% of ducks were bled from the wing vein. The serum was seperated and kept at $-20^{\circ}$C. Afterwards, the ducks were divided into 4 main groups.

3.1 Group I 100 ducks were inoculated intramuscularly with 1 ml of DP-L. Afterwards, the ducks were further subdivided into 3 subgroups (about 30 ducks per subgroup)

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3.1.1 Subgroup 1 (Single vaccination) At fortnightly and monthly intervals, upto 3 months after vaccination, the ducks were sampled for bleeding (about 20-30% of the ducks each time) and for testing their level of the protective immunity (not less than 5 ducks each time). Neutralization and indirect hemagglutination tests were used to detect antibody titer in serums. The immunity of the ducks was determined by means of a challenge-inoculation with virulent duck plague virus.

3.1.2 Subgroup 2 (Double vaccination) About 60 ducks were given their second vaccination 2 months after the first vaccination. Dose, route and titer were the same as in 3.1. At monthly intervals up to 6 months after the second vaccination, the number of ducks sampled was the same as in 3.1.1.

3.1.3 Subgroup 3 (Triple vaccination) About 30 ducks were given their third vaccination 3 months after the second vaccination. Dose, route and titer were the same as in 3.1. At monthly intervals upto 3 months after the third vaccination, the number of ducks sampled was the same as in 3.1.1.

3.2 Group 2 100 ducks were inoculated intramuscularly with 0.5 ml of BEI-DP. Afterwards, the ducks were further subdivided into 3 subgroups and the same procedure followed as in 3.1.

3.3 Group 3 100 ducks were inoculated subcutaneously with 1 ml of BEI-DPM. Afterwards, the ducks were further subdivided into 3 subgroups and the same procedure followed as in 3.1.

3.4 Group 4 100 ducks were unvaccinated. The number of ducks sampled was the same as in the vaccinated groups.

4. <u>The Protective Immunity Test</u>

Each duck was given an intramuscular injection of 1 ml of virulent duck plague virus containing $10^{3}$ $DLD_{50}$. The ducks were observed daily for 2 weeks and the ensuing mortality rate recorded. To exclude nonspecific mortality, all ducks dying within the first 2 weeks were examined and the cause of death was diagnosed by finding

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typical lesions of duck plague. Blood samples were collected from ducks that had survived the challenge administered 14 days into the postinoculation period. Serums were collected and examined by the neutralization and indirect hemagglutination tests.

5. Neutralization Test

All of the serums collected were examined to determined the antibody titer an by $\alpha$ and $\beta$ neutralization tests.

5.1 $\alpha$ Neutralization (constant serum, variable virus) (41,42)

The serums collected were inactivated at 56 C for 30 minutes before use. An initial dilution of 1:10 serum was made with PBS. 96 well flat-bottomed microtiter plates were used throughout. To 0.05 ml of the tested serum, an equal volume of 10-fold serial dilutions of duck plague attenuated virus was added. The serum-virus mixtures were incubated at $37^{o}$C for 1 hour, and 0.1 ml (1x$10^{6}$ cell/ml.) freshly prepared CEF was added to the wells. As a control, the virus was treated in the same way, with the exception that PBS was added instead of serum. After an incubation period of 5 days at $37^{o}$C in a 5% $CO_2$ humidified air atmosphere, the plates were observed CPE in each well under an inverted microscope. The neutralization index (NI) was calculated by subtracting the $log_{10}$ virus titer with serum from the virus titer with PBS.

5.2 $\beta$ neutralization (constant virus, variable serum) (50,52)

Two fold dilutions of serums were prepared in PBS in 96 well flat-bottomed microplate. The first dilution was 1:2 and the

volume in each well was 0.05 ml. An equal volume of medium, containing 100 $TCID_{50}$ of the duck plague attenuated virus, was added to each dilution of serum. As for the incubation period, the amount of CEF used and other conditions, these were the same as in 5.1. The neutralization titer was determined from the highest dilution of serum causing a neutralization of infectivity.

6. Indirect Hemagglutination Test (IHA)

About 50% of serums in each group of vaccine were tested for antibody titer by this method, after choosing the optimal conditions for sensitization.

6.1 Antigen Preparation (81)

Confluent layers of CEF prepared in a Roux-bottle were inoculated with duck plague attenuated virus. After incubation at $37^{\circ}C$ for 1 hour, cell cultures were washed with PBS, then covered with maintainance medium. Three days later, after 80% CPE was observed, the cell suspension was frozen and thawed immediately for 3 cycles. Coarse cellualr debris was sedimented by 30 minutes of centrifugation at 2,000 g at $4^{\circ}C$. Supernatant fluid was concentrated by ultracentrifugation at 100,000 g at $4^{\circ}C$ for 2 hours. The pellet was resuspended with PBS equal to 1/100 of the original volume and stored in aliquots at $-70^{\circ}C$ until use. The protein concentration was determined by Lowry's method (79).

6.2 Preparation of material for IHA

6.2.1 Erythrocyte (75)

Sheep blood was drawn into Alsever's solution (appendix II). Three-to-five-day-old erythrocyte was washed 3 times with saline. From the last sediment, a 10% suspension was prepared in saline. To one part of sheep red blood cells, one part of 7.5% formalin in saline (appendix II) was added slowly, the mixture being stirred constantly. After being held overnight (18-20 hours) at 37 °C in a water bath, the brown-colored cells were washed 4 times with saline. The pack cells were resuspended to 2% suspension with PBS pH 7.2.

2% formalinized red blood cell was mixed with the same volume of 1:40,000 tannic acid in saline (appendix II). After 30 minutes incubation at 37 °C, the cells were washed twice in PBS pH 7.2 and resuspended to 2% suspension in PBS pH 7.2.

6.2.2 Diluent

PBS pH 7.2 containing 0.25% bovine serum albumin was used as a suspending medium for storage of the sensitized cells, and as a diluent for the serum titration.

6.3 Sensitization of sheep red blood cells

The following steps were followed to ensure the optimal conditions for sensitization.

6.3.1 Concentration of duck plague virus antigen

Equal volumes of antigen at various concentrations (9.5-94.5 ug/ml) and 2% tanned, formalinized sheep red blood cell

were mixed and incubated at 37°C for 1 hour, occasionally shaken and then washed 2 times with PBS and resuspended to 1% in diluent.

6.3.2 Duration of sensitization

To 1 ml of 2% tanned, formalinized sheep red blood cell was added an equal volume of a suitable concentration of duck plague virus antigen. The incubations were all carried out at 37°C. The cell suspension was incubated for 30, 60 and 120 minutes with occasional mixing, then washed 2 times and resuspended to 1% in diluent.

An optimal concentration of duck plague antigen and the optimal incubation period were used in the subsequent experiment.

6.3.3 Various cell concentrations

Sensitized, tanned, formalinized sheep red blood cells were suspended at various concentrations (0.5-1.5%) to find the most distinct pattern in terms of sensitivity.

6.4 Indirect hemagglutination procedure

Serums were heat-inactivated and absorbed with an equal volume of 10% formalinized sheep red blood cell at 37°C for 2 hours. Plastic microtitration plates with 96 "V"-shaped wells were used. Serial two-fold dilutions of specimens tested were prepared in a 0.025 ml of the diluent with microtiter dilutor. 0.025 ml of the sensitized cells suspended in the diluent were added to each well. The plates were shaken again and incubated at room temperature. The hemagglutination patterns were read 2 hours later.

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For each run, positive and negative control should be included. Moreover, fixed, non-sensitized cells were added to tested serum to check for the removal of sheep cell agglutinins and both sensitized and non-sensitized sheep cells were added to diluent alone to check for auto-agglutination.

6.5 Positive and Negative control

Positive control was pooled from postchallenging serums that have NI value over 3.0 whereas negative control was collected from prevaccinating serums that have NI value less than 0.5.