CHAPTER 2

MATERIALS AND METHODS

1. Materials

1.1 Organisms: Lyophilised *Pasteurella multocida* was obtained from Division of Veterinary Etiologics, Department of Live stock, Ministry of Agriculture and Cooperatives. This strain was isolated from the outbreak of fowl cholera in ducks in Cholburi on March 26th, 1980 and is the seed culture of fowl cholera vaccine prepared from Department of Live stock nowadays.

1.2 Experimental animals

1.2.1 Rabbits : six adult rabbits weighed about 2.5 to 3.0 kg were used

1.2.2 Mice : young, white, female Swiss mice, weighing 18 to 20 grams were used in this study

1.3 Media

1.3.1 Bacto - Dextrose Starch Agar (Difco Laboratory, U.S.A.)

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

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

1.3.4 Bacto - Nutrient Agar 1.5% (Difco Laboratory, U.S.A.)

1.3.5 Bacto - Tryptic Soy Agar (Difco Laboratory, U.S.A.)

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

1.3.7 Bacto - Tryptose Broth (Difco Laboratory, U.S.A.)

1.3.8 Yeast extract - proteose peptone - cystine agar (YPC agar)[9]: The composition of the medium was as follows :

Yeast extract (Difco)	5.0 g
Proteose peptone no.3 (Difco)	15.0 g
L - Cystine	0.5 g
Glucose	2.0 g
Sucrose	2.5 g
Sodium sulfite	0.2 g
Potassium dihydrogen phosphate	4.0 g
Powdered agar	15.0 g
Distilled water	1,000 ml
pH 7.2	

1.4 Chemicals

1.4.1 Acetone GR (E.Merck Co., Germany)

1.4.2 Alcohol 95% (The Government Pharmaceutical Organization, Thailand)

1.4.3 Albumin bovine, crystallized (Sigma Chemical Company)

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

1.4.5 Collodion 2% in amyl acetate (Sigma Chemical Company U.S.A.)

1.4.6 Complete Freund's adjuvant (Difco Laboratories)

1.4.7 Disodium hydrogen phosphate (May and Baker Ltd., England)

1.4.8 Folin Ciocalteu's phenol reagent (BDH Lab. Reagents)

1.4.9 Formalin (Mallinckrodt)

1.4.10 Incomplete Freund's adjuvant (Difco Laboratories)

1.4.11 Methanol GR (E.Merck Co., Germany)

1.4.12 Phosphotungstic acid GR (E.Merck Co., Germany)

1.4.13 Sodium chloride (May and Baker Ltd., England)

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

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

1.4.16 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 Cover glasses

1.5.6 Erlenmeyer flasks (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 pipet

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 tubes (Pyrex, U.S.A.)

1.5.15 Stirring rod

1.5.16 Syringe (Surgical Manufacturing Co, Ltd.)

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

1.6 Instruments

1.6.1 Analytical balance H6T (E.Mettler, Switzerland)

1.6.2 Autoclave (American Sterilizer Company, U.S.A.)

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 Hot plates (Chromalox, U.S.A.)

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

1.6.8 Klett - Summerson Photoelectric Colorimeter (Klett Mfg. Co., U.S.A.)

1.6.9 Light Microscope (Hertel & Reuss, West Germany)

1.6.10 Millipore filter set

1.6.11 Oven, Precision model 27 (Precision Scientific Co., U.S.A.

1.6.12 Refrigerator (Marco)

1.6.13 Slow - speed stirring motor, ē glass rods (Precision Scientific Co., U.S.A.)

1.6.14 Transmission Electron Microscope (Hitachi HU - 12 A)

1.6.15 UV spectrophotometer (Pye Unicam Ltd., England)

1.6.16 Vaccuum suction

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

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

1.7 Preparation of solution

1.7.1 0.25% formalinized saline

Full - strength formalin (37.40%)	2.5 ml
Sodium chloride	8.5 g
Distilled water	997.5 ml

1.7.2 2.5% sodium chloride

Sodium chloride	2.5 g
Distilled water	100.0 ml

1.7.3 Phosphate buffered saline (0.1 M, pH 7.7)

This buffer was prepared from two stock solutions with the following formular :

Stock solution A (0.2 M Sodium acid phosphate solution)

Sodium acid phosphate, anhydrous	8.0 g
Distilled water, q.s.	1000.0 ml

Stock solution B (0.2 M Disodium phosphate solution)

Disodium phosphate, anhydrous	8.47 g
Distilled water, q.s.	1000.0 ml

Dissolved and stored each stock solution separately, then mixed 1 part of solution A and 9 part of solution B, and diluted the mixture with equal volume of saline solution containing 1.7% weight by volume of sodium chloride in distilled water. The so prepared buffer finally contained 0.1 M phosphate and 0.85% weight by volume of sodium chloride at pH 7.7

1.8 Others

1.8.1 Copper grid, square 400 mesh (Agar Aids, England)

1.8.2 Dialysis tubing (Union Carbide Corp.)

1.8.3 Fuji film for electron microscope (Fuji Films, Japan)

1.8.4 Hemagglutination trays

1.8.5 Kodak photographic paper (Kodak Ltd., England)

1.8.6 Millipore membrane 0.45 μ

1.8.7 Needle

1.8.8 Pinted scissors (A.Dumont & Films)

1.8.9 Surgical blades (Swann - Morton)

1.8.10 Tweezers (Agar Aids, England)

2. Methods

2.1 Negative staining for electron microscopy in capsule study [42]

2.1.1 Preparation of collodion grids

- A dish of about 20 centimeters (cm) in diameter was filled with cold distilled water.

- A large circle of fine wire gauze (about 100 meshes/inch^2) was placed on the bottom of the dish.

- A number, about 20, of 400 meshes grids were placed on the wire gauze.

- Two drops of 2% solution of collodion in amyl acetate were allowed to fall on the central surface of the dish from a dropping pipette.

- After the solvent had evaporated, a solid film was removed from the surface with a forcep. The purpose was to clean the water surface.

- A second film was now formed in the same way and it could be coated on grids.

- The wire gauze, which carried the film - coated grids was lifted from the dish and allowed to dry.

- Put the collodion grids in the vacuum evaporator to make carbon replica.

2.1.2 Preparation of bacterial suspensions of *Pasteurella multocida*

The bacterial cells were grown on 5 solid media: (1) dextrose starch agar, (2) tryptic soy agar, (3) tryptose agar, (4) Yeast extract - proteose peptone - cystine agar (YPC agar) and (5) nutrient agar, incubated for 24 hours (hr) at 37 °C. The growth culture was washed off from agar surface with normal saline (0.85% NaCl) to the required number of cell suspension.

2.1.3 Electron stain

One drop of bacterial suspension was mixed with equal volume of 2% solution, pH 6.5, phosphotungstic acid. The prepared 400 meshes carbon - collodion coated grids were touched to the prepared bacterial suspensions, and the excessive fluid was removed by filter paper. After drying, the grids were examined in a Hitachi HU - 12 A transmission electron microscope at 75 KV and photographed.

The average size of capsule and cell of organism cultured on each medium was measured from 10 cells at x 15,000 magnification.

2.2 Preparation of whole cell antigen and capsular polysaccharide antigen of *Pasteurella multocida*

2.2.1 Preparation of whole cell antigen

2.2.1.1 Bacterin [43]

The microorganisms were grown on 300 milliliters (ml) Difco tryptose agar in Roux bottle and incubated for 24 hr at 37 °C. The growth culture was washed off from the agar surface with 0.25% formalinized saline. This bacterial suspension was then diluted to contain 1 x 10^9 cells per ml.

The bacterial suspension was heated at 56 °C, 1 hr in water bath and was used for active immunization in rabbits as a whole cell antigen or bacterin for the production of antiserum.

The whole cell antigen was inoculated in fluid - thioglycollate media for sterility test and examined for contamination. Any contaminated culture, if found, was discarded.

2.2.1.2 Agglutinating antigen [40]

The antigen consisted of organisms which was grown on Difco tryptose agar, incubated for 24 hr at 37 °C and resuspended in

normal saline to a density approximately equal to 10^9 organisms per ml.

2.2.2 Preparation of capsular polysaccharide antigen [41]

Bacterial cells were grown on 300 ml. Difco tryptose agar and incubated for 24 hr at 37 C. The growth culture was washed off from the agar surface with 2.5% weight by volume (w/v) aqueous solution of sodium chloride, to give a final suspension containing about 10^{10} organisms per ml.

The bacterial suspension was centrifuged at 3500 g. for 30 minutes (min.). The cell - free crude saline extract was obtained by filtering the supernatant through Millipore filter (pore size 0.45 micrometer (μm))

Solvent fractional precipitation for separation of capsular polysaccharide antigen from crude saline extract antigen was performed by adding three volumes of methanol to the crude extract for the first precipitation step and allowed to stand overnight at 4°C. After removal of the precipitate, by decantation or centrifugation, 0.75 volume of acetone was added to the 75 percent methanol supernatant and the precipitate was recovered. It was then dried by solvent evaporation in vacuum suction, and was finally redissolved in normal saline to give a stock solution containing 100 milligram per milliliter (mg/ml).

2.3 Rabbit immunization [43,44]

2.3.1 Production of anti - whole cell serum

Three rabbits were bled before immunization for collection of control normal sera.

The whole cell antigen was administered intravenously in amounts of 0.1, 0.3, 0.5, 1.0 and 2.0 ml respectively at day 1, 4, 7, 10

and day 13 to each of three rabbits. Rabbits were bled before each immunization (at day 4, 7, 10 and day 13) and at day 21, 30, 79, 91 and day 132 after the first immunization. Pooled sera were titered by tube agglutination against whole cell antigen preparation.

2.3.2 Production of anti - capsular polysaccharide serum

Three rabbits were bled before immunization for collection of control normal sera.

The capsular polysaccharide antigen was emulsified with an equal volume of complete Freund's adjuvant and administered intramuscularly in amounts of 1 milligram per kilogram (mg/kg) body weight in first dose. After that the antigen was emulsified with an equal volume of incomplete Freund's adjuvant and administered subcutaneously in amounts of 2,4,4,6 mg/kg body weight, respectively, to each of three rabbits at day 8,15,22 and day 29. Rabbits were bled before each immunization (at day 8,15,22 and day 29) and at day 49,78,89 and day 133 after the first immunization. Pooled sera were titerred against the prepared capsular antigen by indirect hemagglutination.

2.4 Immunoglobulin preparation

Each of the two immune sera, (anti - whole cell sera bled at day 30 from 3 rabbits and anti - capsular polysaccharide sera bled at day 50 from 3 rabbits) was concentrated and partially purified in order to obtain immunoglobulins by precipitating with 50% final concentration of ammonium sulfate at 4°C. The precipitation process was repeated until the precipitate was cleared of hemoglobin. The final precipitate was dissolved in 0.01 Molar (M) phosphate buffered saline (PBS) pH 7.7 at the half volume of original serum, and dialyzed against this buffer at 4°C, 3 days (until it was free of sulfate).[43,44]

The protein content of the two immunoglobulins were determined by the method described by Lowry et al. (45)

2.5 Determination of antibody titer

2.5.1 Agglutination tube tests (40)

To 0.5 ml of two fold dilutions of anti - whole cell serum, equal volumes of prepared antigen were added, and tubes were incubated at 37°C for 2 hr.

Antiserum titers were recorded as the last tube showing complete agglutination. The tubes were then placed in the refrigerator overnight, and the result was re - observed.

2.5.2 Indirect hemagglutination tests (15,41,44,46)

The 2 ml of capsular polysaccharide antigen (500 µg/ml) was added to 2 ml of 5% volume by volume (v/v) suspension of washed human " O " erythrocytes, the cells and capsular polysaccharide were placed in the incubator at 37°C for two hours. The red cells were then separated by centrifugation and washed once with 10 ml of normal saline. Then, 1% horse serum in saline solution was added to the sensitized red cells to produce a 1% suspension. The test was carried out in the multi - hole perspex plates.

Testing antisera were adsorbed with human " O " red cells before use, using 0.5 ml packed cells to each 0.5 ml serum and the antisera was separated by centrifugation. Two fold serial dilutions of antisera in normal saline were prepared and equal volumes of sensitized erythrocytes were added and mixed.

Controls for the normal serum and erythrocytes alone were included by using pre - immunizing sera instead of immune sera and normal red cells instead of sensitized red cells.

The plates were shaken and left at room temperature and the result was observed after 2 hr. The plates were then placed in the refrigerator overnight, and the result was re - observed. Positives were denoted by well marked, agglutination spread over the bottom of holes.

2.6 Mouse passive protection test [43]

2.6.1 Determination of the 50% Lethal Dose (LD_{50}) of P. multocida in mice

The microorganism was cultured on blood tryptose agar and incubated for 24 hr at 37°C. The culture was washed off with normal saline. This bacterial suspension was then diluted in two fold serial dilution. Viable count, pour plate method was made in tryptose agar for each bacterial dilution.

Bacterial suspension was diluted in two fold serial dilution with normal saline to contain 2.5 x 32, 2.5 x 16, 2.5 x 8, 2.5 x 4 and 2.5 x 2 cells/ml. 0.4 ml of each bacterial concentration was inoculated intraperitoneally in each group of ten mice and mortality was observed for 72 hr.

LD_{50} was calculated by the method of Litchfield et al. [47]

2.6.2 Protection tests in mice [43]

2.6.2.1 Determination of the protective dose of antiserum

The amount of 9.1, 18.2 and 36.3 mg of immune globulin of anti - whole cell globulin in 0.25, 0.5, 1.0 ml volume respectively were given subcutaneously in each group of 20 mice and challenged with prepared culture intraperitoneally 24 hr later.

Challenged cultures were inoculated on blood agar for 24 hr at 37°C, and washed off from the agar surface with normal saline, giving an amount of about 30 LD_{50}.

The control group of 20 mice was performed in the same manner, excepted that normal saline was used instead of immune globulin. Deaths and survivals were recorded 72 hr after challenge.

The amount of 7.4, 14.8 and 29.6 mg of immune globulin of anti - capsular polysaccharide globulin in 0.25, 0.5, 1.0 ml volume, respectively, were performed and processed in the same manner as anti - whole cell globulin.

2.6.2.2 Passive protection tests

Selected dose of each immune globulin obtained was used in passive protection tests. 9.1 mg of immune globulin of anti - whole cell globulin and 29.6 mg of anti - capsular polysaccharide globulin were given subcutaneously in each group of 25 mice, 24 hr later all mice were challenged intraperitoneally with 30 LD_{50} of prepared fresh culture.

Control group of 25 mice were performed in the same manner as 2.6.2.1; deaths and survivals were recorded 72 hr after challenge.