CHAPTER III

MATERIALS AND METHODS

MATERIALS

1. Microorganisms

Lyophilized *P. multocida* vaccine strain serotype 8:A (Pm-vac) was obtained from Division of Biological Product, Department of Livestock, Ministry of Agriculture and Cooperation; *P. multocida* 3005 strain and 3007 strain, the isolates from poultry, were from Dr. Kriengsag Saitanu. The 2T35 and 3T5 mutant strains were temperature sensitive (t_s), 2/1U2 and 2/1U3 mutant strains were ultraviolet resistant, and 1S15 and 1S24 mutant strains were streptomycin dependent (strep-d). The 2T35, 2/1U2 and 2/1U3 were developed from 3005 parental strains; 3T5 was developed from 3007 parental strain, 1S15 and 1S24 were developed from Pm-vac parental strain. These mutant strains derived from parental strains which treated with N-methyl-N′-nitro -N-nitrosoguanidine (20), were obtained from Animal Vaccine Research Unit, Chulalongkorn University.

2. Experimental Animals

Ducks : One hundred and forty of ten-day old Kaki Cambell ducks were used.

Rabbits : The immune sera were produced in sixteen young adult New Zealand white rabbits weighing about 2-4 kg each.

3. Media

Brain heart infusion broth (Difco, U.S.A)

Tryptose blood agar base (Difco, U.S.A)

4. Chemicals

Absolute ethanol (Merck, Germany)

Acetic acid (Merck, Germany)

Acrylamide (BRL, U.S.A)

Amido black (Merck, Germany)

Ammonium persulfate (Biorad, U.S.A)

Bis-acrylamide (Sigma, U.S.A)

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

Bovine serum albumin standard (Biorad, U.S.A)

Bromophenol blue (Biorad, U.S.A)

4- Chloro-1-napthol (Sigma, U.S.A)

Chloroform (Merck, Germany)

Citric acid (Mallinckrodt, U.S.A)

Coomassie brilliant blue R-250 (Biorad, U.S.A)

Coomassie brilliant blue G-250 (Biorad, U.S.A)

Disodium hydrogen phosphate (BDH, England)

EDTA: Disodium ethylenediamine tetra acetate dihydrate (Sigma, U.S.A)

Fetal calf serum (Gibco, U.S.A)

Formaldehyde (Carlo erba, Italy)

Freund's adjuvant complete (Difco, U.S.A)

Freund's adjuvant incomplete (Difco, U.S.A)

Glycerol (Merck, Germany)

Glycine (Sigma, U.S.A)

Goat anti-rabbit IgG enzyme HRP conjugate (Zymed, U.S.A)

HEPE : N-(2-hydroxyethyl) piperazine-N'- (-2-ethanesulfonic acid)
(Sigma, U.S.A)

Hydrogen peroxide (Merck, Germany)

Hydrochloric acid (Merck, Germany)

N-laurylsakosine (Sigma, U.S.A)

2- Mercaptoethanol (Biorad, U.S.A)

Methanol (Merck,Germany)

N,N′-methylene bis acrylamide (BRL, U.S.A)

Nitric acid (May & Baker, England)

Paraformaldehyde (Carlo elba, Italy)

o- Phenylenediamine (Zymed, U.S.A)

Phenol (BDH, England)

Potassium chloride (May & Baker, England)

Potassium dihydrogen phosphate (Merck, Germany)

Potassium dichromate (Biorad, U.S.A)

Potassium thiocyanate (Merck, Germany)

Protein molecular weight standard (Biorad, U.S.A)

Silver nitrate (Biorad, U.S.A)

Sodium acetate (Sigma, U.S.A)

Sodium carbonate (Merck, Germany)

Sodium chloride (Merck, Germany)

Sodium dodecyl sulphate (Sigma, U.S.A)

Sodium hydrogen carbonate (Sigma, U.S.A)

Sodium hydrogen phosphate (May & Baker, England)

Sodium hydroxide(May & Baker, England)

Streptomycin sulphate (Sigma, U.S.A)

Sulphuric acid (Merck, Germany)

TEMED (Promega, U.S.A)

Thimerosal (Kecks, U.S.A)

Trismabase (Sigma, U.S.A)

Tris- hydrochloride (Sigma,U.S.A)

Tween 20 (Merck, Germany)

Xylene (Mallinckrodt, U.S.A)

5. Glassware

Beaker (Pyrex, U.S.A)

Centrifuge tubes (Pyrex, U.S.A)

Erlenmeyer flasks (Pyrex, U.S.A)

Funnel

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

Glass dish

Glass plates for SDS-PAGE 16x20 cm, 18x20 cm (Biorad, U.S.A)

L-shape rods

Measuring cylinders (Pyrex, U.S.A)

Measuring pipettes

Pasteur pipettes

Petri dishes (Pyrex, U.S.A)

Stirring rods

Syringes

Test tubes (Pyrex, U.S.A)

6. Instruments

Analytical balance (Sartorius, U.S.A)

Autoclave (Hirayama, Japan)

Bench-Top centrifuge (Clay adams, U.S.A)

Deep freezer -85°c (Forma Scientific, U.S.A)

Freezer -20°c (Tropical Ariston, Italy)

Freezedryer (FTS , U.S.A)

Hamilton syringe (Hemilton, Switzerland)

Hot air oven (Precision, U.S.A)

Hot plate

Incubator (Memmert, Germany)

Light box (Spectroline, U.S.A)

Light microscope (Olympus, Japan)

Magnetic sterrer (Thermolyne, U.S.A)

Microplate Reader (Biorad, U.S.A)

Micropipette (Labsystem, Finland)

Millipore filter set (Gelman Science, U.S.A)

Multichanel micropipette (Labsystems, Finland)

pH meter (Beckman, U.S.A)

Power supply model 1000/500 and model 200/2.0 (Biorad, U.S.A)

Protein II xi Vertical Electrophoresis Cells (Biorad, U.S.A)

Refrigerator (Hitachi, Japan)

Refrigerated centrifuge (Hitachi, Japan)

Refrigerated microcentrifuge (Sigma, U.S.A)

Shaker incubator (New Brunswick Scientific, U.S.A)

Shaker water bath (Julabo, Germany)

Sonicator (Heat System-Ultrasonic Inc., U.S.A)

Stainless cages

Trans-Blot SD Semi-Dry Transfer cell (Biorad, U.S.A)

Ultracentrifuge (Beckman, U.S.A)

Vaccuum suction (Gast, U.S.A)

Vortex cyclomixer (Vortex-genis, U.S.A)

7. Others

Aluminum foils

Centrifuge tube plastic 50, 500 ml (Nalgene, U.S.A)

Dialysis tubing and clips (Medicap, England)

Disposible gloves

Disposible syringes

Floating rack

Microtiter plates (Nunc, Denmark)

Microtube plastics (Treff Lab, Switzerland)

Nitrocellulose membrane 0.2 μ (Micron Separation Inc., U.S.A)

Plastic box

Stainless racks

Surgical blades

Tray

Whatman filter paper No.1 (Whatman, England)

METHODS

1. Virulence and Protective Immunity Study of Mutant Strains of *P. multocida*

The parental strains and mutant strains of *P. multocida* were tested for avirulence and the level of protection in ten day olds of ducks. Eight groups of 15 ducks each were used in vaccination and one group of 20 ducks was used as control. Each group of ducks was injected by intramuscular route at the thigh with 0.1 ml of about 10^{10} CFU/ml of *P. multocida* Pm-vac, 3005, 2T35, 3T5, 2/1U2, 2/1U3, 1S15 and 1S24. The mortality and survival of vaccinated ducks were monitored everyday for 14 days. The control group and survival ducks in each vaccinated group were challenged with Pm-vac strain by intramuscular route at day 14 after vaccination. The challenge dose was 1.77×10^{9} CFU/duck, the mortality and survival rate of ducks were observed everyday for 7 days after challenge. The bacteria used for vaccination and challenge were diluted ten fold in normal saline solution (NSS) and determined for total number of bacteria by plate counting on tryptose blood agar (TBA, Difco).

2. Preparation of Antigen Extracts of *P. multocida* and Protein Determinations

2.1 Preparation of Organisms

The cultures of *P. multocida* Pm-vac and 3005 parental strains, and 2T35, 2/1U2 and 1S24 mutant strains were used for preparation antigen extracts. The purpose for using these three mutant strains as antigen was to compare the properties

of the three groups; low virulence and high protection (2T35), high virulence and high protection (2/1U2), low virulence and low protection (1S24). These strains were thawed from stock cultures, kept at -80°c and streaked for isolation on TBA plates. Isolated colony was streaked on surface of TBA plates and incubated at 37°c for 24 hr. Cells were harvested by washing cultures on agar surface with NSS and suspension were adjusted to contain $1x10^9$ cells/ml. The suspension of *P. multocida* were inoculated in a flask of 400 ml brain heart infusion broth (BHI, Difco), 2 flasks for each antigen extract, then incubated in shaker incubator (New Brunswick Sci, U.S.A) at 37°c for 24 hr. Cells were harvested from the broth and washed three times with phosphate buffer saline (PBS), pH 7.2 by centrifugation at 2,000xg for 30 min at 4°c (91, 92).

2.2 Preparation of Potassium Thiocyanate (KSCN) Antigen Extracts

Bacterial cells were suspended in 10 ml of 0.5 M potassium thiocyanate (KSCN) in 0.08 M sodium chloride, pH 6.3 and slowly stirred in shaker water bath (Julabo, Germany) at 37°c for 5 hr. This suspension were centrifuged at 19,000xg for 30 min at 4°c to pellet the cells. The supernatant was dialyzed against four times changes of 0.1 M tris-hydrochloride, 0.32 M sodium chloride buffer, pH 8.0 for 48 hr at 4°c to remove any possible dialyzable fragment (29, 92).

2.3 Preparation of Capsule Extracts

Bacterial cells were suspended in 10 ml of 0.04 M sodium chloride and slowly stirred in shaker water bath at 56°c for 1 hr. Cells were removed by centrifugation at 19,000xg for 30 min at 4°c. The supernatant was dialyzed against one change of 0.15 M sodium chloride at 4°c for 48 hr (34).

2.4 Preparation of Outer Membrane Protein (OMP) Extract

Bacterial cells were suspended in 10 ml of 10 mM HEPE buffer , pH 7.4 and disrupted by using a sonicator (Heat System Ultrasonic Inc., U.S.A). Cell debris was removed by centrifugation at 2,000xg for 30 min at 4°c. The supernatant was centrifuged using ultracentrifuge (Beckman, U.S.A) at 100,000xg for 60 min at 4°c. The pellet which contained total membrane was resuspended in 2 ml of 2% sodium lauryl sarcosinate (Sakosyl) detergent in 10 mM HEPE buffer, pH 7.4 and incubated at 22°c for 60 min. The sodium lauryl sarcosinate detergent-insoluble outer membrane enriched fraction was sedimented by centrifugation at 100,000xg for 60 min at 4°c and then washed two times with distilled water. The pellet was dissolved in distilled water and dialyzed against distilled water for 48 hr (93, 94).

2.5 Lipopolysaccharide (LPS) Extract

Bacterial cells were suspended in 10 ml of TAE buffer (40 mM tris acetate, pH 8.5 with 2 mM EDTA) and mixed with 20 ml of alkaline solution containing 3 gm of sodium dodecyl sulfate (SDS), 0.6 gm of trismabase and 6.4 ml of 2 N sodium hydroxide in 100 ml of distilled water. The mixture was heated at 55-60°c for 70 min and then mixed with phenol-chloroform (1:1, v/v). The supernatant of the mixture after centrifugation at 16,000xg for 10 min was mixed with 10 ml of 3 M sodium acetate, pH 5.2. LPS was precipitated by adding two volumes of ethanol. The precipitate was dissolved in 20 ml of buffer solution containing 50 mM tris-hydrochloride, pH 8.0 and 100 mM sodium acetate and re-precipitated with two volumes of ethanol. The final precipitate of LPS was dissolved in distilled water (95).

All of antigen extracts were concentrated by freeze-drying using lyophilizer (Freeze-Dryer FTS System, U.S.A). Antigens were aliquoted and stored at -20°c until use.

2.6 Protein Determinations of *P. multocida* Antigen Extracts

The protein contents of antigen extracts were determined by dye-binding method described by Bradford (96). Bovine serum albumin (BSA) was prepared for standard protein at the concentrations of 20, 40, 60, 80 and 100 µg/ml. Standard BSA and antigen extracts in a volume of 160 µl were added in each well of microtiter plates. For dye-binding reaction, 40 µl of dye reagent

(Coomassie brilliant blueG-250, Biorad) was added to each well. The mixture was mixed immediately and left at room temperature for 5-60 min. The absorbance was measured at wavelength 595 nm by using Microplate Reader model 450 (Biorad, U.S.A). The protein contents were estimated from standard curve.

3. Analysis of Antigen Extracts of *P. multocida* by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The protein patterns of KSCN antigen, capsule, OMP extract and patterns of LPS extract of *P. multocida* Pm-vac , 3005, 2T35, 2/1U2 and 1S24 were analyzed by SDS-PAGE with discontinuous buffer according to the method described by Laemmli, 1970 (97).

3.1 Preparation of Vertical Slab Gels

3.1.1 Preparation of Separating Gels

The slab gels were 1.0 mm in thickness and 16 cm in width. The gels of 7.5%, 10% and 15% separating gel were prepared in the lower part from 30% stock solution of acrylamide and N,N′-methylene-bis-acrylamide of the ratio 30:0.8. The gels were prepared in 0.375 M tris-hydrochloride pH 8.8, and contained 0.1% sodium dodecyl sulfate (SDS) and the gels were polymerized chemically by the addition of 0.2% of N,N,N′,N′-tetramethylethylene diamine (TEMED) and 10% ammonium persulfate, polymerizing agent. The gels were mixed by gently swirling

and poured into the assembled gel sandwich about 11 cm, and were overlayed on the top with distilled water. The gels were allowed to polymerize overnight at room temperature.

3.1.2 Preparation of Stacking Gels

The stacking gels were consisted of 4% acrylamide in 0.125 tris-hydrochloride pH 6.8, 0.2 % SDS and the gels were polymerized chemically by the addition of TEMED and ammonium persulfate. The prepared gels were filled into assemblied gel sandwich with a comb of fifteen identical wells 2.0 cm length inserted in the upper part at a length of 5 cm and then the gels were allowed to polymerize at room temperature at least 1 hr.

3.2 Preparation of Samples

Antigen extracts were prepared in the sample buffer of 0.6 M tris-hydrochloride pH 6.8 containing 2% SDS, 10% glycerol 5% 2-mercaptoethanol and 0.5% bromophenol blue as the dye. Sample concentrations were 15 µg/well combined in equal volume of sample buffer and boiled at 95°c for 4 min.

3.3 Molecular Weight Protein Markers

The broad range reference marker protein standards (Biorad, U.S.A) used for indicating the molecular weights (MW) of the protein bands were aprotonin (MW 6,500), lysozyme (MW 14,500), soybean trypsin inhibitor (MW 21,500), carbonic anhydrase (MW 31,000), ovalbumin (MW 45,000), bovine serum albumin (MW 66,000), phosphorylase B (MW 97,400), β-galactosidase (MW 116,000) and myosin (MW 200,000), prepared 1 part in 19 part of sample buffer and boiled at 100°c for 5 min.

3.4 Gel Loading

The comb was slowly removed from the gel. Each well was rinsed with electrophoresis buffer. Using a 25 or 50 μl Hamilton syringe, 10-15 μg protein/well of sample was gently loaded beneath the buffer in each well. Every well was loaded with the same volume of each sample. The molecular weight protein markers were loaded in the first lane or among the sample lanes. If the well was not needed, the same volume of sample buffer was loaded instead.

3.5 Electrophoresis

The electrophoresis was carried out by using the protein II xi vertical electrophoresis cells (Biorad, U.S.A). The electrophoresis buffer pH 8.3 containing 0.025 M tris, 0.192 M glycine and 0.1 % SDS. The buffer of 2,000 ml was put into the lower buffer tank and 400 ml into the upper buffer tank reservoir. The power (Biorad Power Supply model 1000/500, U.S.A) was set at 40 mA per one gel and the current was kept constant. The time taken to run the gel was about 4 hr or until the tracking dye was migrated about 10 cm from the top of separating gel.

3.6 Procedure for Staining

3.6.1 Coomassie Brilliant Blue R-250

The gels were fixed in the fixing solution containing 40% methanol with 10% acetic acid for 30-60 min and stained with 0.1% Coomassie brilliant blue R-250 in 40% methanol, 10% acetic acid for at least 2 hr. The gels were then destained in the destaining solution until the protein bands were seen and the background were clear.

3.6.2 Silver Stain

The gels of LPS extracts were stained by silver stain (98). The gels were placed into 40% methanol with 10% acetic acid (v/v) fixing solution in a clear glass dish for 30 min and fixed in 10% ethanol with 5% acetic acid (v/v) for 15 min two times. The gels were oxidized with potassium dicromate with nitric acid for 5 min, washed with deionized water several times until all the color were removed from the gels and stained with 0.1% silver nitrate for 20 min, washed with deionized water 1 min. The gels were developed with developer containing 3% sodium carbonate and 0.019% paraformaldehyde for 30 sec, the developer was replaced and gels were developed until the LPS bands were seen. The developer was drained off and reaction was stopped with 5% acetic acid.

3.7 Determination of Molecular Weights

The relative mobility (Rf) of proteins were determined and calculated from the formula

$$Rf = \frac{\text{distance of the protein migration}}{\text{distance of the tracking dye migration}}$$

The Rf were plotted against the known MW of protein markers in the semilog scale and the MW of the unknowns were estimated from standard calibration curve.

3.8 Storage of Stained Gels

After staining completed, gels were photographed on a bright light box (Spectroline, U.S.A) and stored after drying on cellophane membrane. For drying a gel, two sheets of cellophane of the size bigger than the gel were soaked in water. One sheet of cellophane was placed onto a glass plate and stained gel was placed carefully avoiding air bubbles. The second sheet of cellophane was placed over the gel and air bubbles were removed. Cellophane was folded back to each edge of the glass plate and was fixed with clips to prevent the cellophane moving when it dried. The gel was left to dry at room temperature or 37°c for 24 hr.

4. Preparation of Rabbit Immune Sera and Determination of Antibody Titer

Among the organisms described in materials, 4 strains of *P. multocida* were used. *P. multocida* 2T35 mutant strain was avirulent and provided good protection in ducks, 1S24 was nonvirulent mutant strain, Pm-vac and 3005 serotype 8:A were parental strains. These strains were used for preparation of immune sera. Each of two New Zealand white rabbits (2-4 kgs) were used for each immunization program. Rabbits were bled before immunization for collection of rabbits control normal sera. All of rabbit immune sera were collected before each immunization or 7-10 days after immunization and each boost (99). Immune sera were separated from blood by centrifugation and the supernates were removed, aliquoted and stored at -20°c or -80°c. The antibody titers were determined by ELISA.

4.1 Preparation of Rabbit Immune Sera Against Whole Cell Antigens

The suspensions of *P. multocida* Pm-vac, 3005, 2T35 and 1S24 containing $1x10^9$ cells/ml were prepared in 0.25% formalinized saline (v/v), heated at 56°c for 1 hr in water bath. The bacterial suspensions were checked for survival bacterial cells by streaking on tryptose blood agar. Each whole cell antigen was administered intravenously at marginal ear vein in amount of 0.1-2.0 ml at days 0, 7, 14, 21, 35 and boost at day 70.

4.2 Preparation of Rabbit Immune Sera Against Sonicated Cell Antigens

The suspensions of *P. multocida* Pm-vac, 3005, and 2T35 containing $1x10^9$ cells/ml were prepared in 0.25% formalinized saline (v/v), heated at 56°c for 1 hr in water bath and sonicated using sonicator until cells were broken completely. Each sonicated cell antigen was administered subcutaneously in amount of 0.1-1.0 ml four doses, 1 week interval and boost at day 35. For the first dose at day 0 antigen was emulsified with an equal volume of complete Freund's adjuvant and the following doses were mixed with an equal volume of incomplete Freund's adjuvant.

4.3 Preparation of Rabbit Immune Sera Against KSCN Antigen Extract and Capsule Extract

The KSCN antigen and capsule extract of *P. multocida* 2T35 were suspended in 0.25% formalinized saline (v/v), administered via subcutaneous route at the neck in amount of 1 mg/ml four times, 1 week interval and two boosts at day 35 and 56. Each antigen extract was emulsified with an equal volume of complete Freund's adjuvant for the first dose at day 0. The antigen was emulsified with an equal volume of incomplete Freund's adjuvant for the following doses.

4.4 Determination of Antibody Titers by Enzyme-Linked Immunosorbent Assay (ELISA)

Antibody titers of rabbit immune sera were determined by using ELISA. The 96 wells microtiter plates (Nunc, Denmark) were coated with homologous KSCN antigen extract 100 µl in each well at 1 µg/well in 0.1 M carbonate-bicarbonate coating buffer, pH 9.6. After incubation in moist chamber at 4°c overnight, the plates were washed three times for 3 min each with phosphate buffered saline with 0.05% tween 20 pH 7.4 (PBS-T) and then non specific binding sites were blocked with 3% bovine serum albumin (BSA) in PBS-T 100 µl/well. After incubation at 37°c for 1 hr, plates were washed with PBS-T three times 3 min each. Each ten fold dilution ($1:10^1$-$1:10^6$) of rabbit immune sera were diluted in 1% BSA in PBS-T and filled (100 µl/well) into the duplicate wells for antibody titer determination. After

incubation at room temperature for 1 hr, plates were washed as above and 100 µl of goat anti rabbit immunoglobulin conjugated to horseradish peroxidase (Zymed, U.S.A) diluted at 1:3,000 with 1% BSA in PBS-T were added in each well and the plates were incubated at room temperature for 1 hr. After the plates were washed, o-phenylenediamine substrate solution with hydrogen peroxide were added (100 µl/well) and the plates were incubated at room temperature for 15-20 min in the dark. The enzymatic reactions were stopped by adding 50 µl of 4 N sulfuric acid to each well. The absorbances were measured at wavelength 492 nm by using Microplate Reader model 450 (Biorad, U.S.A). The antibody titer was the highest dilution of immune sera giving absorbance 0.5 compared to absorbance of blank.

5. Western Blot Analysis of Antigen Extracts of *P. multocida* with Rabbit Immune Sera (100)

5.1 Western Blot Transfer

The KSCN antigen extract, capsule, OMP and LPS extract of *P. multocida* Pm-vac parental strain and 2T35 mutant strain were separated by SDS-PAGE on 7.5% polyacrylamide gel and on 15% gels for LPS, then transfered onto a 0.2 µ nitrocellulose membrane using semidry eletrophoretic transfer cell (Biorad, U.S.A). The concentration of antigen run on SDS-PAGE were about 200 µg. A comb of 2 wells was used, a large well for antigen loading and a small one for molecular weight protein markers. Following electrophoresis the gels were

equilibrated in 48 mM tris-hydrochloride, 39 mM glycine, 20% methanol and 0.04% SDS transfer buffer pH 9.2 for 15 min to remove of eletrophoresis buffer salts and detergents. Nitrocellulose membrane and filter papers were wet by slowly aligning into transfer buffer, allowing to soak for 15-30 min. Ten sheets of pre-soaked filter paper were placed onto the platinum anode, the pre-wetted nitrocellulose was then placed on top of the filter papers and the equilibrated gel was placed next on top of the nitrocellulose membrane, aligning the gel on the center of the membrane. A ten sheets of pre-soaked filter papers were placed on top of the gel, for all between steps carefully removing air bubbles. The semidry electrophoretic transfer cell was assembled and run for 2 hr at 25 V. After the transfer was finished, the gel was removed and stained with Coomassie brilliant blue or with silver stain for LPS to determine that the proteins were completely transfered. Efficient binding of antigen to the nitrocellulose membrane was determined by staining a portion of the membrane for sample proteins and molecular weight protein standard with 0.1% amido black in 40% methanol with 10% acetic acid at least 1 hr.

5.2 Immuno - analysis of Antigen with Rabbit Immune Sera

The nitrocellulose was cut into strips for immunodetection. The strips were washed in 0.5% BSA in tris buffered saline (TBS) pH 7.4 (50 mM tris-hydrochloride with 0.85% sodium chloride) and non specific binding sites were blocked with 3% BSA and 10% calf serum in TBS pH 7.4 for1 hr at room temperature. Each two strips were probed with rabbit immune sera against whole cell

Pm-vac, 3005, 2T35, 1S24, the sera against sonicated cell Pm-vac, 3005, 2T35, the sera against KSCN antigen extract 2T35 and the serum against capsule extract 2T35. Immune sera were diluted 1:5 in 1% BSA in TBS pH 7.4, rabbit normal sera were used as control. Antigen and sera were incubated at 4°c overnight. After washing with TBS pH 7.4 several times, the strips were blocked as above for 15 min at room temperature. The nitrocellulose strips were incubated with 1;1,000 diluted goat anti - rabbit immunoglobulin conjugated with horseradish peroxidase at room temperature for 2 hr and washed several times with TBS pH 7.4 to remove the unbound conjugate. The nitrocellulose strips were then incubated in substrate (4-chloro-1-napthol) with hydrogen peroxide diluted in TBS pH 9.5 substrate buffer at room temperature for 1 hr. The specific bands observed were dark violet in color. The enzymatic reaction was stopped by washing the strips in distilled water. The nitrocellulose membranes were air dried and photographed immediately after development. The storage was done by wrapping in aluminum foil to protect from light.