Chapter III

Methods

1. Physiological and biochemical characteristics

All *P. multocida* strains were streaked on TSB and examined for the physiological characteristics of the colonies after 18 to 24 hrs at 37 C. The colonies were then streaked on MacConkey agar and SS agar and examined for the ability of the organisms to grow on the media. The isolated colonies were also inoculated in 2 ml of BHI broth and incubated for 6 hrs at 37 C. These young cultures were used to study the biochemical characteristics of the organisms.

Fermentation reactions were determined in phenol red broth base containing 1% glucose, xylose mannitol, lactose, sucrose, maltose, mannose, arabinose, dulcitol and 10% lactose. Two drops of young cultures was inoculated in the medium. The results were recorded after 24, 48 hrs and up to 7 days.

The ability of growth in salt was performed by inoculation of one drop of young cultures in the nutrient broth without salt and with 6% NaCl. The turbidity in tube indicated the ability to grow in salt. Recorded the reactions after 24 hrs at 37 C.

The temperature of growth was tested in BHI broth. One drop of young cultures was inoculated in the

medium and immediately incubated at 25 C, 37 C. and 42 C. Recorded the results after 24 hrs of incubation.

The ability of organisms to produce acid and acetoin from glucose fermentation was tested in MR-VP medium. One drop of young cultures was inoculated in the medium. After 24 hrs of incubation, the VP reagent was dropped in the MR-VP culture. Recorded the pinkish-red colour on the surface of the medium as positive result. The methyl red reagent was dropped in the 48 hrs culture and recorded the red colour.

The indole test was determined by inoculation of one drop of young cultures in peptone broth. After 24 hrs of incubation, the cultures were tested with Kovac reagent and recorded the red colour at the surface of the medium as positive reaction.

The production of nitrite was performed by inoculation of one drop of young cultures in nitrate broth. After 24 hrs of incubation, the cultures were tested with nitrate reagent and recorded the red colour as positive reaction.

The esculin hydrolysis test was done in esculin broth. One drop of young cultures was inoculated and recorded the black colour in the tube after 24 hrs at 37 C.

The decarboxylase test was examined in the decarboxylase medium containing 1% arginine, lysine and ornithine. Three drops of young cultures were inoculated

in the medium and the control (no amino acid in basal medium).The surface of the medium was overlaid with 1-2 ml of sterile liquid paraffin and recorded the results after 24 hrs at 37 C. The purple colour in tube test and yellow colour in tube control were recorded as positive result.

The reaction on TSI medium was determined by stabbing the colonies into the butt through the bottom of the tube and streaking on the slant. Recorded the reaction in the butt and slant after 24 hrs at 37 C.

The utilization of citrate as the sole source of carbon for metabolism was performed on Simmon citrate medium. The colonies on TSB were streaked on the slant of the medium and recorded the growth with an intense blue colour on the slant after 24 hrs of incubation.

The ability of the organisms to split urea and form ammonia was tested on Urea Christensen's medium. The colonies on TSB were streaked on the slant and recorded the pink-red colour on the slant after 24 hrs of incubation.

The fermentative or oxidative reaction was performed by stabbing two tubes of OF medium to the depth about 1/2 inch. Overlaid one tube with soft paraffin. Result was recorded after 24 hrs and up to 2 wks. The fermentative result was shown by the yellow colour in both tubes, only yellow colour in the tube without paraffin

indicated oxidative reaction. The motility test was also observed in the OF medium tubes.

The catalase test was done by dropping 1-2 drops of 3% H_2O_2 on the colonies from DSA and observing for the bubble as positive test.

The oxidase test was done by dropping 1-2 drops of oxidase reagent directly on a few suspected colonies and observed the purplish-black colour which developed within 10 sec.

The gelatin hydrolysis test was done by spotting the colonies on the gelatin plates. After 48-72 hrs of incubation, flooded the plate with acid mercuric chloride solution and recorded the clear zone around the colony as positive result.

Capsules were observed by Jasmin method (13) The colony was suspended in a loopful of Jasmin reagent on a clean slide. The dried film was fixed in methyl alcohol, drained and flamed to burn off the excess alcohol. The preparation was then stained with crystal violet or safranin for 30 sec to 1 min. The stain was washed off with water, dried the slide and examined for the capsules under oil immersion lens.

2. Procedures for the identification of capsular and somatic serotypes

2.1 Serological procedure for the identification of capsular serotypes

A indirect hemagglutination test (IHA) for the identification of capsular serotypes A,B,D, and E was described previously by Carter (68) with minor modification (99).

2.1.1 Preparation of capsular typing antisera type A,B,D and E

0.3 ml of each antigen serotype A,B,D, and E was injected intravenously at the ear of each rabbit. The antigen doses were gradually increased and injected intravenously every other day (Table 6). After 9.1 ml of antigens were administered, withdrew the blood from marginal vein and checked for the titer of antisera. Whenever sufficient titer (at least 1:1024) was obtained, the heart blood was collected and the antisera were separated from the clot and stored frozen. They were inactivated for 30 min at 56 C before used.

2.1.2 Preparation of antigen extracts of P. multocida

The organisms which were stored in skim milk at -85 C were inoculated on TSB and incubated overnight at 37 C. After subculture for once, the colonies were cultivated in 2 ml of BHI broth for 6 hrs at 37 C and

Table 6 Injected doses of capsular antigens

Types \ Volumes (ml)	Times of injections (every other day)														
	1	2	3	4	5	6	7	8	9	10 a	11	12	13	14 b	15 c
A	0.3	0.4	0.5	0.6	0.8	1.0	1.0	1.5	1.5	1.5	1.5	1.5	0.5	0.5	0.5
B	0.3	0.4	0.5	0.6	0.8	1.0	1.0	1.5	1.5	1.5	-	-	-	-	0.5
D	0.3	0.4	0.5	0.6	0.8	1.0	1.0	1.5	1.5	1.5	1.5	1.5	0.5	0.5	0.5
E	0.3	0.4	0.5	0.6	0.8	1.0	1.0	1.5	1.5	1.5	-	-	-	-	0.5

a = Checked the titers, 5 days after the 10th injection.

b = Checked the titers, 7 days after the 14th injection.

c = Withdrew the heart blood 1 week after the last injection.

0.5 ml of each young culture was grown on DSA. The 18 hrs growth was harvested with 4 ml of sterile 0.85% sodium chloride solution. It was heated at 56 C for 30 min to assist the removal of the capsular antigen. The organisms were then separated by centrifugation for 30 min at 1,000 g. The supernatant was transferred to another tube and kept frozen.

2.1.3 Preparation of sensitized cells

Human packed red blood cells group 0 were washed three times with PBS pH 7.2. and 0. 1 ml of packed cells from the last washing for 10 min at 600 g were added to 1.5 ml of antigen extracts. After mixed thoroughly, they were then incubated in a waterbath for 2 hrs at 37 C with shaking at intervals. The sensitized cells were separated and washed twice with PBS. The washed cells were adjusted to 0.5% suspension with PBS.

2.1.4 Performance of IHA test

Two fold serial dilutions of the antisera type A,B,D,and E were made in 0.25 ml of PBS in U bottom microtiter plates. The dilution was started from 1:2 to 1:1024 for a screening test. In case of positive reaction, the test should be further diluted to 1:32,768. An equal volume of sensitized cells was added to each dilution. The last two wells of the row were negative controls. They were: (1) sensitized cells control; PBS was mixed with sensitized cells, (2) red cells control;

diluted serum 1:2 was mixed with unsensitized cells. The microtiter plates were shaken and incubated in a moist chamber for 2 hrs at 25 C before the results were read. The IHA titer was expressed as the reciprocal of the highest dilution of serum showing uniform layers of red cells evenly lining the bottom of the wells. Dense red buttons at the bottom of the wells indicated the absence of agglutination.

If there was any agglutination in the negative control wells, the test should not be interpreted and the cells should be discarded.

If there was any agglutination in the red cells control well, the agglutinin in serum should be absorbed by using an equal volume of 10% unsensitized cells mixed with the antiserum and incubated for 2 hrs at 37 C with shaking at intervals. The serum was removed by centrifugation for 10 min and the cells were washed twice with PBS. These cells were ready to use for the preparation of sensitized cells.

If there was any doubtful reading, the second one was done after the plate was kept overnight in the refrigerator.

2.1.5 Titration of capsular typing antisera

The titers of capsular typing antisera type A,B,D, and E were determined by making a two fold serial dilution of antisera starting from 1:2 to

1:32,768. Human washed packed red cells group O were sensitized with homologous antigen type A,B,D, and E. The IHA test was performed as described earlier.

The IHA antisera titer was the highest dilution that showed positive result.

2.2 Non-serological procedures for the identification of type A and D

2.2.1 Identification of type A using staphylococcal hyaluronidase (71)

The test was conducted on TSB and DSA. Each strain examined was streaked transversely about 3-5 mm wide across the whole plate. S. aureus strains producing hyaluronidase enzyme were streaked heavily at right angles to the pasteurella streak line. The plates were incubated overnight at 37 C. The hyaluronidase effect of type A was manifested as a diminution of the pasteurella growth at the intersection with staphylococcal streak.

2.2.2 Identification of type D strains of P.multocida with acriflavine (11)

The colonies of P. multocida were inoculated in 3 ml of BHI broth. The cultures were incubated for 18-24 hrs at 37 C. The broth was centrifuged for 30 min at 1,000 g and 2.5 ml of the supernatant was removed and discarded. A 0.5 ml of 1:1,000 aqueous solution

of acriflavine neutral was then added to 0.5 ml of broth containing the organisms. After mixing to resuspend the organisms, the tube was left stationary at room temperature. If the strain was type D, a heavy flocculent precipitate became evident within 5 sec. After 30 min, the heavy precipitate settled, leaving a distinct supernatant.

2.3 Serological procedure for the identification of somatic serotypes (73, 76)

2.3.1 Preparation of somatic typing antisera type 1,3, 3x4, 12, and 13

Each antigen was injected subcutaneously (Table 7)at subscapular region of each rabbit once a week. The first injection was antigen with complete Freund's adjuvant and subsequent injections were antigen with incomplete Freund's adjuvant. If sufficient titer was obtained, the heart blood was collected and the serum was separated from clot and stored frozen.

2.3.2 Preparation of heat stable antigen

Ninety-one strains of P. multocida from stock culture media were cultivated on TSB for 18-24 hrs at 37 C. The isolated colonies were subcultured on TSB three times in order to make them lose their capsules. The typical colonies were inoculated in 2 ml of BHI broth for 6 hrs at 37 C. A 0.5 ml of young cultures was heavily seeded on DSA and incubated for 24 hrs at 37 C. The growth

Table 7 Injected doses of somatic antigens

	Times of injections (once a week)						
Volumes (ml) / Types	a 1	b 2	b 3	b,c 4	b,d 5	b,e 6	b,f 7
1	1	1	1	1	1	1	1
3	1	1	1	1	1	1	1
3x4	1	1	1	1	1	1	1
12	1	1	1	1	1	1	1
13	1	1	1	1	1	1	1

a = Antigen with complete Freund's adjuvant

b = Antigen with incomplete Freund's adjuvant

c = Checked the titers 5 days after the 4th injection.

d = Antisera of serotypes 3x4, 12 and 13 were collected.

e = Checked titers of antisera of serotypes 1 and 3.

f = Withdrew the heart blood 1 week after the last injection

was harvested with PBS pH 7.0 containing 0.3% formalin. The suspension was boiled in the waterbath for 1 hr at 100 C. The supernatant was removed after centrifugation for 30 min at 1,000 g. The supernatants, which were heat stable antigens, were stored frozen,

2.3.3 Performance of GDPT

There were two kinds of antisera used in the GDPT. One was the reference antisera obtained from the National Animal Disease Control, Iowa. USA. The other was the somatic typing antisera prepared for this study.

2.3.3.1 Performance of GDPT using reference typing antisera

The agar gels consisted of 1% agarose in 8.5% NaCl. Melted agar 18 ml was poured onto 84x94 mm glass plate. The gels were cut by using the LKB template and telescopic well punchers. The well was 4.0 mm in diameter and 75 mm apart from center to center. Reference antiserum 25 μl was placed in center well and an equal volume of heat stable antigens was placed in outer wells. The glass plates were incubated at 37 C in a moist chamber. Recorded the precipitin line after 24, 48 and 72 hrs.

The lyophilized reference typing antisera were reconstituted with PBS pH 7.0 and checked for the titers

as described in 2.3.3.3 before used, 1% agarose in 8.5% NaCl was used and the system was performed at 37 C.

2.3.3.2 Performance of GDPT using somatic typing antisera

The agar gels consisted of 1% agarose in PBS pH 7.0 (100). The gel was performed in the same manner as mentioned above. Heat stable antigens 25 µl was placed in central well and an equal volume of somatic antisera type 1,3,3x4,12, and 13 was placed in the outer wells. The glass plates were incubated at 25 C in a moist chamber. Recorded the precipitin line after 24, 48 and 72 hrs.

2.3.3.3 Titration of somatic typing antisera

Eighteen milliliter of 1% agarose in PBS pH 7.0 was moulded on the glass plate. Two fold serial dilution starting from 1:2 to 1:32 of homologous heat stable antigens were prepared. The antigen 25 µl was then placed in central well and an equal volume of undiluted, 1:2, 1:4, 1:8, 1:16, and 1 : 32 of somatic typing antiserum was placed in the outer wells. Recorded the sharp precipitin line after incubation at 37 C for 24, 48 and 72 hrs as the titer of that somatic typing antiserum. The titer that used as somatic typing antisera was of one dilution above the antisera titer.

3. The analysis of protein patterns of P.multocida whole cell sonicated extracts by SDS-PAGE.

3.1 Whole cell preparation

Seventeen strains of P. multocida stored in skim milk at -85 C were cultivated onto TSB for initial growth. The plates were incubated overnight at 37 C. Isolated colonies were picked up and subcultured on TSB. After 18 hrs of incubation, six typical colonies of P. multocida were inoculated into 5 ml of BHI broth and incubated for 6 hrs at 37 C with shaking at 100 min^{-1}.Each young culture 2.5 ml was then transferred to 250 ml of BHI broth and further incubated for 20 hrs at 37 C with shaking at 100 $min.^{-1}$ The late log phase cultures were harvested by centrifugation for 30 min at 10,000 g in a swing-bucket rotor (RPR-9-2) at 4 C. The pellets were washed twice with sterile 0.145 M NaCl solution of the same volume as the broth culture used. The washed packed cells were weighed out and kept frozen at -85 C.

3.2 Preparation of sonicated extracts

The frozen washed packed cells were thawed and an equal volume (W/V) of sterile 0. 145 M NaCl solution was added. The suspensions were sonicated by a Handy Sonic Model UR-20P for 15 min with 5 min intervals. The system was placed on ice to keep it cool.Approximately

90% cell breakage was obtained as examined by gram stain. The intact bacteria and debris were removed by centrifuging for 1 hr at 10,000 g in a swing-bucket rotor (RPR-20-3) at 4 C. The supernatant fluids which were designated as sonicated extracts were separated and divided into small aliquots and stored frozen.

3.3 Determination of protein concentration of sonicated extracts.

Protein concentration was determined by the method of Lowry et al (101). The stock reagents consisted of reagent A, 2% Na_2CO_3 in 0.1 N NaOH; reagent B,2% sodium potassium tartrate and reagent C, 1% $CuSO_4.5H_2O$. The working reagent D was prepared by thoroughly mixing 100 ml of reagent A with each milliliter of reagent B and C. Reagent A, B, and D must be freshly prepared. BSA at the concentration of 12.5, 25, 50 and 100 μg/ml was used for preparing a standard curve, All unknown samples were diluted with distilled water to make a concentration between 10-100 μg/ml. Each 2 ml of reagent D was mixed with 1 ml of each BSA concentration or diluted unknown sample. The tubes were left for 10 min at room temperature. Reagent E 120μl was added to each tube and immediately mixed. After 30 min, the absorbance was read in the spectrophotometer at wavelength 650 nm. The blank was prepared in the same fashion as the unknown except

distilled water was used instead of the protein solution. The optical density of each standard was then plotted against the concentration on an arithmetic graph paper. The optical density of each unknown was finally calculated from the slope of this standard curve.

3.4 Performance of SDS-PAGE

The protein patterns of the whole cell sonicated extracts of selected P. multocida strains were analysed by SDS-PAGE with discontinuous buffer according to the method described by Laemmli (92).

3.4.1 Preparation of vertical slab gel

The slab gels were 1.5 mm in thickness. They consisted of 10% resolving gel in the lower part and 3% stacking gel in the upper part. The gels were prepared from 30% stock solution of acrylamide and bis-acrylamide of the ratio 30:0.8. The gels were polymerized chemically by the addition of 0.025% by volume of TEMED and ammonium persulfate. The resolving gel of 10% acrylamide and a length of 12.5 cm contained 0.375 M Tris-HCl pH 8.8 and 0.2% SDS. It was allowed to polymerize overnight in a refrigerator before moulding the stacking gel. The stacking gel of 3% acrylamide and a length of 2 cm contained 0.125 M Tris-HCl pH 6.8 and 0.2% SDS was polymerized chemically in the same way as for the resolving gel except it was left at room temperature for 2

hrs after inserting a comb of twenty identical wells. Before pouring and polymerizing the gel,the solutions were degased thoroughly.

3.4.2 Preparation of protein samples

Protein samples were prepared in the sample buffer of 0.0625 M Tris-HCl pH 6.8 containing 2% SDS, 10% glycerol, 5% 2- mercaptoethanol and 0.1% bromophenol blue as the dye. The final protein concentration was approximately 2.5 mg/ml and the ratio of SDS: protein was 4:1 (91). The samples were boiled at 100 C for 5 min. They were divided into aliquots and kept frozen.

3.4.3 Molecular weight markers

The reference marker proteins (102) used for indicating the molecular weight of the protein bands were carbonic anhydrase (MW 29,000), egg albumin (MW 45,000) bovine plasma albumin(MW 66,000),phosphorylase B (MW 97,400), β-galactosidase (MW 116,000) and myosin (MW 205,000). All the reference marker proteins gave rise to one band except the myosin which demonstrated a second band remaining at the origin.

3.4.4 Loading

The samples were boiled at 100 C for 2 min before loading. Each lane contained 35 μg of protein per sample.The molecular weight markers were loaded in the second lane or among the sample lanes. The first lane was loaded with bromophenol blue.

3.4.5 Electrophoresis

The electrophoresis was carried out by using the LKB 2001-001 vertical electrophoresis unit. The electrode buffer pH 8.3 contained 0.025 M Tris,0.192 M glycine and 0.1% SDS. The buffer was put into the lower buffer tank and the upper buffer reservoir. The power was set at 60 mA per two gels and the current was kept constant. During the electrophoresis, the temperature was also kept constant at 10 C. The time taken to run the gel was 3.30 hrs and the tracking dye was about 1-2 cm to the bottom of the gel.

3.4.6 Procedure for staining

The gels were fixed in the fixing solution (103) for at least 30 min and then stained with Coomassie blue G250 for 2 hrs. The gels were then destained in the destaining solution until the protein bands were seen and the background was clear.

For preserving the gels, the destained gels were soaked in the gel dryer solution containing 40% methanol and 5% glycerol for at least 2 hrs. They were dried in the LKB 2003 slab gel dryer for 3.30 hrs or longer until the gels were dried.

3.4.7 Determination of molecular weights

To determine the mobility of a protein, it was calculated from the formula (104) :

Mobility = $\frac{\text{distance of protein migration}}{\text{length after destaining}} \times \frac{\text{length before staining}}{\text{distance of dye migratio}}$

The mobility was plotted against the known molecular weight of protein markers on semilogarithmic paper and the molecular weights of the unknowns were estimated from this calibration curve.