Chapter IV

Results

1. Bacteriological characteristics

Bacteriological characteristics of P. multocida were identified according to the characteristics described by Weavers et al as shown in table 2 (17).

1.1 Physiological characteristics

Colonies from ninety-one strains of P. multocida on TSB (Table 8) were glistening, smooth, entire edge and convex. Colonies of type A (Figure 2) and D were mucoid. No hemolysis was observed on TSB. They were cultivated aerobically and produced a distinctive smell which was helpful in recognition. The sizes of colonies varied from 0.5 to 2.5 mm in diameter dependent on their capsular types, i.e. colonies of type A were the largest whereas colonies of type B (Figure 3) and E were smaller. It was revealed by gram stain that they were gram negative coccobacilli with capsules which were seen as halos around the cells. Their capsules were also demonstrated by Jasmin method (13). With safranin, the organisms were stained dark orange and the capsules were colourless halos around the organisms whereas the background was light pinkish orange. Fewer capsules were seen in most reference somatic strains. They arranged in singly. Pleomorphism in which short rods or

filaments were seen in some type D strains. They were non-motile and non-spore forming.

They could not be cultivated on MacConkey or SS agar.They could neither be grown in nutrient broth without salt nor with high salt concentration. They could be grown at both 25 C and 37 C. Most strains especially the strains isolated from poultry could be grown at 42 C.

1.2 Biochemical characteristics

The biochemical characteristics of P. multocida strains are shown in table 9. The reaction on TSI was demonstrated as acid slant and acid butt without producing gas or hydrogen sulfide. Nitrate was reduced to nitrite. Indole and urease were not produced. MR-VP tests were negative. Neither gelatin nor esculin was hydrolysed. Ornithine decarboxylase test was positive. Lysine decarboxylase test and arginine dihydrolase test were negative. Citrate was not utilized. Catalase and oxidase enzymes were presented.

They were fermentative in which a small amount of acid was produced in the OF tube without sterile paraffin. Acid was detected by the fermentation of glucose, mannose, and mannitol without producing gas. Sucrose was fermented by most strains but three strains isolated from pigs were not. Maltose, lactose and 10% lactose were not fermented. Acid from xylose was fermented by all pig strains and some

human strains, but most poultry strains as well as vaccine 8:A strain were not. Arabinose was fermented by almost all poultry strains and a few human strains, but rarely by pig strains. Dulcitol was not fermented by any strains except reference somatic strains type 1 and 14.

2. Serological typing by the Carter-Heddleston System

The typings were performed with those organisms that had already been identified as P. multocida. Any non-P. multocida strains were rejected from the tests.

2.1 Capsular types

There were no reference antisera used for capsular serotyping by the IHA test. The antisera for typing capsular serotypes A,B,D and E were produced in pasteurella free rabbits. Titration of antisera and specificity of the IHA reaction were determined. Nagative results were obtained when homologous and heterologous antigens were tested with typing antisera A and D (Table 10). According to these results, it was concluded that the typing antisera A and D could not be produced in any rabbits. The capsular serotype A and D, therefore, could not be determined by means of the IHA test. The non-

serological procedures for capsular typing of type A and D were used instead.

However, antisera type B and E could be produced and the titer of each antiserum was 1:8192 against its homologous antigen (Table 10). No cross-reactions were detected when these antisera were tested against reference type A and D antigens. These results proved that antisera type B and E were suitable used as typing antisera in the IHA test since they had high titer and good specificity. Reproducibility was also shown when other lots of antigen extracts were tested.

Capsular type A and D were identified by the non-serological tests of staphylococcal hyaluronidase and acriflavine respectively. The hyaluronidase test for typing the P. multocida type A was examined better on DSA than TSB. The growth of type A was diminished by the action of enzyme at the intersection of P. multocida and staphylococcal streak lines (Figure 4), whereas type B,D, and E were not affected. P. multocida type D was flocculated and precipitated in the acriflavine solution. This reaction was specific for type D only (Figure 5).

The capsular typing of P. multocida strains were therefore typed by the methods of non-serological tests for type A and D and the IHA tests for type B and E. Known capsular type A (P-1059), type B (P-1404), type D (P-3881), and type E (P-1235) were employed as controls. All

reference somatic and vaccine strains were also included in the tests. Type 1,3,5,9,10,12,14,16, CU strain, and 8:A were typed as type A. Type 2 and 6:B were typed as type B. Type 7 was typed as type E. Type 6,8,13 and 15 were untypable (Table 10). As examined under light microscope, fewer capsules were seen among these untypable strains.

All strains of P. multocida isolated from human, pigs and poultry were typed (Table 11). In human, 11 strains (64.71%) were type A;5 strains (29.41%) were type D and 1 strain(5.88%)was untypable.In pigs,19 strains (82.61%) were type A and 4 strains (17.39%) were type D. In poultry, 25 strains (86.22%) were type A and 4 strains (13.78%) were untypable. Type D was not found in poultry. All sixty-nine isolates,therefore,were typed as 55 strains (79.71%) of type A, 9 strains (13.04%) of type D and 5 strains (7.25%) of untypable. Neither type B nor E was detected from the isolates.

2.2 Somatic serotypes

There were two kinds of typing antisera used for somatic serotyping by GDPT. One was reference typing antisera of NADC type 1 to 16 except type 8 and 15. The other was somatic typing antisera type 1,3, 3x4, 12, and 13 which were produced in pasteurella-free rabbits. The somatic typing antiserum type 3x4 was prepared by the immunization of CU strain. Titration and specificity of both kinds of typing

antisera were performed with homologous heat-stable antigens. The specificity of the test was recorded by the sharp precipitin line which located near and concave toward the corresponding antigen well (Figure 6). Any diffuse precipitin line which was near and concave toward the antiserum well was indicated as non-specificity.

The titers of reference antisera used as typing antisera were as follows : antisera type 1 and 14 were 1:4; antisera type 2,3 and 10 were 1:2; and antisera type 4,5, 6,7,9,11,12,13 and 16 were undiluted.

The titers of somatic antisera used as typing antisera were as follows : antisera type 1 and 3 were undiluted; antisera type 3x4, 12, and 13 were 1:8, 1:4, and 1:8 respectively.

Both typing antisera were shown their specificities with their homologous heat-stable antigens. Known reference somatic antigens type 1 (X-73), type 2 (M-1404), type 3 (P-1059), type 5 (P-1702), type 6 (P-2192), type 7 (P-1997), type 9 (P-2095),type 10 (P-2100),type 11 (P-903), type 12 (P-1573), type 13 (P-1591), type 14 (P-2225), type 15 (P-2237), and CU antigen were used as controls. All capsular reference strains and vaccine strains 6:B and 8:A were also included in the GDPT (Table 12).

2.2.1 Somatic serotypes as determined by reference typing antisera

Using reference typing antisera, type A was type 3; type B and E were type 2; and type D was type 3,12. CU strain was type 3,4; 8:A strain was type 1; and 6:B was type 2 (Table 12).

All strains of P. multocida isolated from human, pigs and poultry were typed by reference typing antisera (Table 13). In human, 6 strains (35.30%) were type 1; 4 strains (23.54%) were type 3,4; 3 strains (17.64%) were type 3; 2 strains (11.76%) were type 3,4,12; and 2 strains (11.76%) were untypable. In pigs, 14 strains (60.88%) were type 3; 6 strains (26.08%) were type 3,4; 3 strains(13.04%) were type 4,7,12. In poultry, 27 strains (93.10%) were type 1 ; and 2 strains (6.90%) were type 3,4.

All sixty-nine isolates therefore were typed as 33 strains (47.82%) of type 1; 17 strains (24.64%) of type 3; 12 strains (17.39%) of type 3,4; 2 strains (2.90%) of type 3,4,12; 3 strains(4.35%) of type 4,7,12 and 2 strains (2.90%) of untypable (Table 13).

Serotype 3 was predominantly found in pigs and serotype 1 was prevalently found in poultry. Serotype 1 was not found in pigs whereas serotype 3 was not found in poultry but both serotypes were commonly found in human. Serotype 3,4 was the second serotype found among some isolates of human, pigs, and poultry.

2.2.2 Somatic serotypes as determined by somatic typing antisera

Although somatic antisera type 1,3, 3x4, 12, and 13 were shown their specificities with homologous strains, non-specific precipitin lines were sometimes observed near and concave toward the antisera wells of type 1 and 3.

Using somatic typing antisera, type A was type 3; type B and E were untypable; type D was also untypable with non-specific precipitin lines 1 and 3. Vaccine strain 6:B was untypable; and 8:A was type 1 with non-specific precipitin lines 1 and 3. Other heterologous somatic reference strains were untypable (Table 12).

All strains of P. multocida isolated from human, pigs, and poultry were typed by somatic typing antisera (Table 14). In human, 6 strains (35.30%) were type 1; 4 strains (23.54%) were type 3,4; 2 strains (11.76%) were type 3; 2 strains (11.76%) were type 1,3; 1 strain (5.88%) was type 3,12; 1 strain (5.88%) was type 1,3,12; and 1 strain (5.88%) was untypable. In pigs,11 strains (47.84%) were type 1,3; 6 strains (26.08%) were type 3; 2 strains (8.69%) were type 3,4; 2 strains (8.69%) were type 1,3,12; 1 strain (4.35%) was type 1,3,4; and 1 strain was type 12. In poultry, 26 strains (89.65%) were type 1; 1 strain (3.45%) was type 3; 1 strain (3. 45%) was type 1,3 and 1 strain (3.45%) was type 1,3,4.

All sixty-nine isolates therefore were typed as 32 strains (46.38%) of type 1; 14 strains (20.29%) of type 1,3; 9 strains (13.04%) of type 3; 6 strains (8.69%) of type 3,4; 3 strains (4.35%) of type 1,3,12; 2 strains (2.90%) of type 1,3,4; 1 strain (1.45%) of type 12; 1 strain (1.45%) of type 3,12; and 1 strain (1.45%) of untypable (Table 14).

Serotype 1;3; and 3,4 were commonly found in human whereas serotype 1 was prevalent in poultry. Similar results were also obtained as examined by reference typing antisera. But serotype 1,3 in pigs were more common than serotype 3 which was predominant when reference typing antisera were used. The serotype 1,3 were also detected in two human strains and one poultry strain but they were not found in any isolates as typed by reference antisera. Serotype 3,4 were detected in pigs but serotype 1 was not. The same results were demonstrated by using reference typing antisera. In poultry, one strain was serotype 3 and one strain was serotype 1,3,4 but no serotype 3,4 were observed. On contrary to the result of reference typing antisera that serotype 3,4 was found in poultry but serotype 3 and 1,3,4 were not.

Using both typing antisera, one antigenic component was found in most strains whereas two or three antigenic components were noted in some isolates. If somatic typing antisera were used, the somatic serotypes

that had more than one antigenic component were mostly associated with serotype 1 and 3 (Table 13 and 14).

2.3 P. multocida serotypes

The serotype of P. multocida strain was based on the difference in its capsular and somatic antigens. The combination of capsular and somatic serotype data when the isolates were identified by reference typing antisera (Table 15) therefore indicated that P. multocida human serotypes were 5 strains (29.44%) of A:1; 3 strains (17.64%) of A:3; 3 strains (17.64%) of A:3,4; 2 strains (11.76%) of D:3,4,12; 1 strain (5.88%) of D:1; 2 strains (11.76%)of D:untypable;and 1 strain(5.88%) of untypable:3,4. P. multocida pig serotypes were 14 strains (60.88%) of A:3; 5 strains (21.74%) of A:3,4; 3 strains (13.03%) of D:4,7,12 and 1 strain (4.35%) of D:3,4. P. multocida poultry serotypes were 25 strains (86.20%) of A:1; 2 strains(6.90%) of untypable: 1; and 2 strains (6.90%) of untypable : 3,4.

Total finding of P. multocida serotypes from sixty-nine isolates were 30 strains (43.48%) of A:1; 17 strains (24.64%) of A:3; 8 strains (11.58%) of A:3,4 ; 3 strains (4.35%) of D:4,7,12,; 2 strains (2.90%) of D:3,4,12; 1 strain (1.45%) of D:1; 1 strain (1.45%) of D:3,4; 2 strains (2.90%) of D : untypable; 3 strains (4.35%) of untypable : 3,4; and 2 strains (2.90%) of untypable:1

When the isolates were identified by somatic typing antisera (Table 16), P. multocida human serotypes were 5 strains (29.44%) of A:1; 3 strains (17.64%) of A:3,4; 2 strains (11.76%) of A:3; 1 strain (5.88%) of A:1,3; 1 strain (5.88%) of D:1; 1 strain (5.88%) of D:1,3 ; 1 strain (5.88%) of D:3,12; 1 strain (5.88%) of D:1,3,12; 1 strain (5.88%) of D:untypable; and 1 strain (5.88%) of untypable : 3,4 P. multocida pig serotypes were 10 strains (43.48%) of A:1,3 ; 5 strains (21.74%) of A:3; 2 strains (8.69%) of A:3,4; 2 strains (8.69%) of D:1,3,12; 1 strain (4.35%) of A:1,3,4; 1 strain (4.35%) of D:3; 1 strain (4.35%) of D : 12, and 1 strain (4.35%) of D : 1,3. P. multocida poultry serotypes were 24 strains (82.75%) of A:1; 1 strain (3.45%) of A:1,3 ; 2 strains (6.90%) of untypable : 1; 1 strain (3.45%) of untypable : 3 ; and 1 strain (3.45%) of untypable : 1,3,4.

Total finding of P. multocida serotypes from sixty-nine isolates were 29 strains (42.02%) of A:1; 12 strains (17.39%) of A:1,3; 7 strains (10.14%) of A:3; 5 strains (7.25%) of A:3,4; 3 strains (4.35%) of D:1,3,12; 2 strains (2.90%) of D:1,3,; 1 strain (1.45%) of A:1,3,4; 1 strain (1.45%) of D:1; 1 strain (1.45%) of D:3; 1 strain (1.45%) of D:12; 1 strain (1.45%) of D:3,12; 1 strain (1.45%) of D:untypable; 2 strains (2.90%) of Untypable : 1; 1 strain (1.45%) of untypable:3 ; 1 strain (1.45%) of untypable:3,4; and 1 strain (1.45%) of untypable : 1,3,4.

By using both typing antisera, P. multocida serotype A:1; A:3; and A:3,4 were commonly found in human whereas serotype A:1 was prevalent in poultry. With reference typing antisera, predominant serotype in pigs was A:3 but incidence of serotype A:1,3 was higher when somatic typing antisera were used. Type D which associated with other somatic serotypes were detected in some human and pig strains but not in poultry.There were no detection of serotype A:1 and D:1 in pigs, or A:3 and D:3 in poultry (Table 15,16 and Figure 7). Type B and E were not detected from any isolates of human, pigs and poultry. However, serotype B:2 was found in reference strains type B and type 2, and vaccine strain 6:B whereas serotype E:2 was found in type E and type 7 was typed as type E:7 (Table 10 and 12).

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

The protein concentration of sonicated extracts from seventeen P. multocida strains were ranged from 286.47 mg% to 1718.75 mg% (Table 17). The electrophoretic patterns of protein of all strains tested, regardless of serotypes and origins, are displayed in figure 8. The molecular weights of the protein bands were estimated from

the calibration curve. Approximately 46-56 protein bands were directly visible among these strains and at least twelve major protein bands were common (Figure 8). These similar protein bands were located at the MWs 150K, 140K, 100K, 85K, 76K, 72K, 58K, 49.5K, 46K, 40K, 36.5K and 29.5K respectively. However, some differences in the position of minor protein bands were particularly apparent among strains. The SDS-PAGE profiles of P. multocida regarding to serotypes showed that certain serotypes had different protein patterns.

For serotype B:2; type B and 6:B strains (Figure 8, lanes K and L respectively), their differences were seen at the electrophoretic mobilities of 150 K to 100 K range, 58 K to 40 K range and 36.5 K to 29.5 K range respectively. In addition, type B possessed an atypical band at the MW 122.5 K (Figure 9, lane K) which could not be found in any other strains.

For serotype A:3,4; CU strain (Figure 8, lane I) and AC-01 strain (Figure 8, lane Q), their differences were shown at the electrophoretic mobilities of 72 K to 49.5 K range, 36.5 K to 29.5 K range and below 29 K range respectively. CU strain had a dense band at MW 32 K (Figure 9, lane I). PM 1002 (Figure 8, lane O) and PM 3011 (Figure 8, lane R) were another two strains of somatic serotype 3,4. Their protein patterns exhibited the

differences at the same electrophoretic mobilities as those serotype A:3,4. However, PM 1002 possessed a dense band at MW 32.5 K (Figure 9, lane O).

For serotype D:3,12; type D had serotype D:3,12 (Figure 8, lane N) and TECH-2 had serotype D:3,4,12 (Figure 8, lane P). Though their serotypes were not exactly the same, they shared two somatic antigen 3 and 12 in common. They both possessed dense bands at the same MWs 66 K and 30.5 K (Figure 9, lanes N and P respectively), but the differences between them were demonstrated at the electrophoretic mobilities of 58 K to 49.5 K range and 36.5 K to 29.5 K range respectively (Figure 8, lanes N and P respectively).

In addition, those strains which possessed somatic serotype 3 associated with serotype 4 or 12 or 4 and 12 (Figure 8, lanes N through R) except CU strain (Figure 8, lane I) had a dense band at MW 66 K. This band of other serotypes were seen as a diffuse or less dense band.

No significant differences were found among the protein profiles of serotype 1 and 3 (Figure 9, lanes B through H) except some variations in intensities of protein bands could be seen.