CHAPTER V

DISCUSSION

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

As shown in the results, each group of ducks vaccinated with live attenuated vaccine showed the differences in mortality and survival after challenge. Ducks vaccinated with Pm-vac and 3005 parental strains died with acute infection within 24 hr. In the groups vaccinated with temperature sensitive and streptomycin dependent mutant strains, ducks died within 1-9 days following vaccination. Some factors such as environment and high dosage of vaccine may influence on the number of deaths and mortality. The dosage used in this experiment of about 10^9 CFU/duck was the same as the dosage of vaccine used in the field. Various concentrations of vaccine used were reported such as 10^9 CFU/ml in ducks (19,23) and rabbits (24), 10^8 CFU/ml in turkeys (15,20) and rabbits (25) and 10^6 CFU/ml in turkeys (21). The mutant strain can be divided into two groups according to the mortality rate after vaccination. Mutant strains of 1S24 and 2T35 were in low virulent group, and 1S15, 2/1U2 and 3T5 mutant strains were in high virulent group. After challenge with virulent Pm-vac, most of ducks were dead at day 1. The high level of protection were observed in ducks vaccinated with 2T35, 3T5, 2/1U2

and lower level of protection in ducks vaccinated with 2/1U3, 1S15 and 1S24. The level of protection may be higher than the results obtained if the challenge dose was less than 10^9 CFU/duck. or the double or three vaccinations was used. In the nature, the infection dose did not exceed 10^9 CFU/duck. The challenge dose of 10^2-10^9 CFU/ml were generally used and 2-3 vaccinations were administered (19-21, 23-25). The data suggested that double vaccination was superior to single vaccination in stimulation to produce effective immunity. The results of high protection (78.57%) and low virulence (6.67%) of the temperature sensitive 2T35 mutant strain was correlated to the result of Yoshida, et al., 1993 (101). They reported that 2T35 provided 62% protection in first vaccination with $1.5x10^5$ CFU/duck of challenge dose and provided 80% protection in second vaccination with $1x10^6$ CFU/duck of challenge dose. Mortality rate of 8% and 2% were observed after first vaccination and second vaccination respectively. The same result was supported from the same laboratory by Saitanu, et al.(102) that 2T35 showed 100% protection after challenge.

The several reports of t_s and strep-d of *P. multocida* developed from live vaccine and virulent strains for improvement of live vaccine efficacy and to decrease mortality of animal received live vaccine have appeared. Results of this thesis suggested that the 2T35 t_s mutant strain can be a live attenuated vaccine candidate.

2. Antigen Extracts and Protein Determination

To analyze various antigen extracts of *P. multocida*, various mutant strains were selected for study in comparison with parental strains Pm-vac and 3005: 2T35, the most interested strain, which showed low virulence and high protection, 2/1U2 which showed high virulence and high protection and 1S24 which showed non virulence and low protection. The studies included the antigenic components, the role in stimulation of immune response in animal, antigen-antibody reaction and protective response of various antigen extracts of various types of bacterial strains. In this regards four types of important surface antigenic components of antigen extracts of *P. multocida* were investigated.

The first component was the KSCN antigen extract which contained protein, cell wall fragment, carbohydrate, lipopolysaccharide, hyaluronic acid, DNA, RNA (104) and cell membrane vesicles (32). KSCN antigen extracts from avirulent and virulent strains were found to be immunogenic and protective against homologous and heterologous challenges (29, 30, 32, 33). Most immunogenic components were found in the protein fraction. However, when KSCN fraction was depleted of LPS, there was a partial loss of immunogenicity (105). The second was capsule antigen extract usually contained 40% protein, 15% carbohydrate and small amount of LPS (28, 34, 103). This fraction has been shown to be immunogenic and nontoxic to mice (106). The capsule composes of hyaluronic acid and acted as the cause of disease (1, 10). The capsule was

considered as a virulence factor of *P. multocida*, because non-encapsulated variants of pathogenic strains were less virulent than encapsulated strains (10).

The third antigen was the outer membrane protein (OMP) extracted by using Sakosyl detergent. The OMP is a part of the outer membrane of the cell wall exposed on the cell surface (36), antigenic determinants of OMP were reported as the target for protective antibodies (107).

The last fraction was lipopolysaccharide (LPS) extract. LPS is an important serological marker in Heddleston's typing system and is the major component of outer membrane of gram negative bacteria (108, 109). LPS of gram negative bacteria consists of three regions, the lipid A, core oligosaccharide and o-specific polysaccharide (110). LPS of *P. multocida* are semirough LPS (63) and directly hemagglutinated chicken and turkey red blood cells (111).

3. Analysis of Antigen Extracts by SDS-PAGE

In this investigation SDS-PAGE was performed to examine protein profiles of KSCN antigen, capsule, OMP and LPS extract. The extracts contained proteins of MW ranging from 25 to 116 kDa. There were variations in intensities and differences in major and minor protein pattern among antigen extracts and among strains of *P. multocida*. By using the same method in analysis of protein from sonicated whole cell of *P. multocida* isolated from human, pigs and poultry, 46-56 protein bands in region between 29 kDa and 150 kDa were reported (112).

Protein profiles of KSCN antigen extracts of *P. multocida* of Pm-vac, 3005, 2T35, 2/1U2 and 1S24 contained similar protein of 38, 58, 61, 63 kDa, but there were differences in intensities in each protein band. Similar molecular weights of protein has been shown to be the membrane protein extractable by KSCN reported by Lu et al (32, 86). The protein profiles contained the 37.5 kDa which was related to 38 kDa. This protein is the predominant OMP in KSCN antigen extract. The protein patterns were quite similar among strains studied. Total proteins in sonicated cells contained similar proteins at of 29.5, 36.5 40, 46, 49.5, 58, 72, 76, 85, 100, 140 and 150 kDa, some differences in the position of minor protein bands were particularly apparent among strains. The SDS-PAGE profile of *P. multocida* regarding serotypes showed that certain serotypes had different protein patterns (112). Protein from sonicated cells of *P. multocida* serotype 1 (X-73) and isolates were similar as reported by Ireland, et al.(113).

The major differences between isolates were in the position of one of the major protein present in the 34-38 kDa. Total proteins of *P. multocida* isolated from turkeys and chickens with fowl cholera (72) had 26 protein bands of MW ranging 14-200 kDa. Most bands were common in all isolates, variation in intensities were seen in the 30-40 kDa in each strain. Major bands of 30-40 kDa were observed in all strains.

The electrophoretic protein pattern of capsule in all strains had similar major protein of 36 and 38 kDa. The similar protein of 58, 60, 63, 77, 85 and 86 kDa were found in all strains but some protein bands were different in intensities. Other investigators suggested that capsule of *P. multocida* P-1059 serotype 3:A from sodium chloride solution extract (2.5 S) contained proteins of MW 43-44 kDa (28). Other report suggested that monoclonal antibody (MAb) positive against 2.5 S antigen of *P. multocida* P-1059 recognized the major 35.5 kDa protein and minor 78 kDa protein (114). The results shown in Figure 3 suggested that profiles of KSCN antigen extracts and capsule extract at 38, 58, 61, 63, 77 and 80 kDa were similar in all strains.

The protein pattern of OMP analyzed on 10% gel and 15% gel revealed major 38 kDa band and minor bands of 29, 31 and 48 kDa. Lee, et al. (72) also reported that the OMP of avian strains migrated in the range of 28-40 kDa. Studies of Lugtenburg, et al. (115) demonstrated that envelop protein profiles of *P. multocida* isolated from swine were protein bands approximately 36-38 kDa. The others (94) suggested that proteins of 34.5 kDa and 38 kDa of *P. multocida*

P-1059 were presented in OMP fraction of in vitro-grown and vivo-passaged organisms. The OMP profile of field isolates expressed 27, 34.5, 38 and 46 kDa. Different strains of the same serotype with variation in the OMP composition has been reported. Lu, et al. (35) found that the OMP of serotype 3:A composed of protein of 24.6, 27, 30.4, 37.5, 49.5 and 64.5 kDa. Kim, et al. (116) analyzed protein profile of OMP of *P. multocida* P-1059 grown in iron-restricted. It was shown that *P. multocida* P-1059 expressed 76, 84 and 94 kDa which were similar to in vivo-grown organism. The proteins of 29, 34.5 and 45 kDa were found in bacteria grown in these conditions and in iron-repleted media.

KSCN antigen extract contained proteins which could be detected in capsule and OMP extracts. The dominant protein of 38 kDa in OMP, in KSCN antigen extract was found to be important immunogen (32, 35, 86, 94).

The SDS-PAGE of LPS extract was observed by using sensitive silver stain on 15 % gels. Polysaccharide of LPS was reactic component (98) and the intensities were depend on side chain of o-specific polysaccharide (95). The LPS band appeared to be the result of clustering of LPS molecules (63). In this study, the result showed that all strains of *P. multocida* expressed LPS at average MW of 7 kDa. Similarly low MW of LPS were found in hemorrhagic septicemia strain at MW of 8.1-14.4 kDa (117), LPS from isolates of *P. multocida* from rabbits contained one or two protein bands migrated faster than protein standard 14 kDa and slower than LPS of *S. mennesota* which contained 4.3 kDa (63). Lee, et al. (118) compared LPS of CU low virulent vaccine to virulent field isolates,

but found no differences between this component. However the mobility of LPS was reported to be correlated to the shortening of the core-oligosaccharide (119). In this investigation, proteins contaminated at the higher MW were observed. These proteins could be removed by digesting with proteinase K and the protein sample boiled and incubated at 60°c for 60 min (114). The confirmation of LPS was done by staining the same separating gel with Coomassie blue, carbohydrate components in LPS were not stained.

4. Determination of Antibody Titers of Rabbit Immune Sera

Enzyme-linked immunosorbent assay (ELISA), a convenient, rapid and more sensitive method was used to determine the antibody titers of rabbit immune sera against various antigens of *P. multocida*. The ten-fold serial dilutions of immune sera were allowed to react with the homologous KSCN antigen extract. The result indicated that all rabbit immune sera reacted strongly with homologous antigen. Avakian, et al. (92) indicated that the suitable coating plate antigens in ELISA were KSCN extract and capsule extract.

In our study, KSCN antigen extract was used for coating the microplates because it contained various cell components that could therefore be capable of detecting antibody in rabbit immune sera against whole cell, sonicated cell, capsule and KSCN antigen extract. The immune response in rabbits were detected at day 21 after the first immunization. The observation by others in

detection of IgG and IgA by ELISA revealed that serum IgG activity was detected at day 17 (32) or day 19 (33). Nasal mucosa IgA activity was detected at day 9 (33) or day 31 after first immunization (32). In our study, high antibody titers of all rabbit immune sera were 10^3-10^6 at day 35-49 after first immunization and reached the highest titer of 10^5-10^6. Antibody titers then were gradually decreased at days 70-90 in some immune sera, and the titer of 10^3-10^5 were still observed. The others (86)reported that hyperimmune sera of rabbit immunized with KSCN antigen extract contained IgA titer of 10 and IgG titer of 409,600.

Chlink, et al. (120), reported that turkey immunized with CU live vaccine showed the significant IgG level increased after vaccination, but IgA level was not significantly increased.

In this study, each type of antigen distinctly stimulated the immune response in rabbits. In Pm-vac strain, the sonicated cell stimulated and produced higher antibody titer than that of whole cell antigen. Sonicated cell of 3005 stimulated and produced antibody titer that was risen faster than those produced by whole cell. Two types of antigens from 2T35 strain, KSCN antigen extract and capsule were similar in stimulating of immune response.

Avakian, et al. (92) used various types of antigens i.e., KSCN extract, capsule, LPS, protein complex, heat-stable antigen, salt soluble antigen and sonicated cells from CU vaccine as coating antigens in ELISA. The results

suggested the antibody titer against KSCN antigen and capsule extract were correlated with survival rate after challenge , more nonsurvivals in low antibody titers and more survivals in high antibody titers. The similar result (118) was reported in chicken sera received double vaccination gave 100% protection against challenge. This indicated that the level of antibody is correlated with protection and can predict the survival rate. KSCN antigen extract was the most immunogen used to immunize animals and resulted in protection against challenge (29, 32, 33, 93).

5. Western Blot analysis of Antigens of *P. multocida* with Rabbit Immune Sera

The results of KSCN antigen extract, capsule, OMP and LPS extract of Pm-vac and 2T35 reacted with rabbit immune sera were observed by the reactive bands of antigen-antibody complexes on nitrocellulose. In this study, KSCN antigen extracts of *P. multocida* Pm-vac were strongly recognized by rabbit immune sera against whole cell Pm-vac, 3005, 2T35, 1S24, sonicated cell Pm-vac, 3005 and KSCN antigen extract 2T35 at protein of 38, 48, 52, 55, 73 and 77 kDa. The reactions of KSCN antigen extract of 2T35 reacted strongly at protein of 29, 35, 36, 38 and 55 kDa to all immune sera except immune serum against capsule 2T35.

Lu, et al. (86) investigated that the hyperimmune sera detected ten different antigens and the four major antigens were 29.9, 38.5, 40.9 and 71 kDa.

In western blot analysis of capsule extract of Pm-vac, protein of 26, 27 and 45 kDa strongly reacted with immune sera against whole cell Pm-vac, 3005, 2T35, 1S24, sonicated cell 3005 and KSCN antigen extract 2T35. Capsule extract 2T35 were more strongly recognized by rabbit immune sera against whole cell 2T35 and 1S24 than with immune sera against sonicated cell Pm-vac and KSCN antigen extract 2T35 at 43 kDa.

In reaction of OMP extract of *P. multocida* Pm-vac revealed that 38 kDa protein were observed with rabbit immune sera against sonicated cell 3005, 2T35 and KSCN antigen extract of 2T35. In reaction of OMP extract 2T35, the 38 kDa protein was recognized with strong reaction by immune sera against whole cell 1S24, sonicated cell Pm-vac, 3005, 2T35 and KSCN antigen extract 2T35, but weak reaction by immune sera against whole cell Pm-vac, 3005, 2T35 and capsule extract 2T35.

The results of western blot analysis of OMP were reported that homologous immune sera against antigen extract recognized major proteins at 27, 37.5, 49.5, 58.7, 64.4 kDa (35), they suggested that OMP immunogen exposed on the cell surface and accesibled by antibodies, since adsorption of immune sera with intact *P. multocida* resulted in a significantly reduction of antibody activities, especially the 37.5 kDa and the 37.5 kDa appeared to be the major protein of OMP. OMP can be a promising vaccine candidate as evaluated in rabbit (120).

Monoclonal antibody 1608 (MAb-1608) that directed against 37.5 kDa (105) can recognize protective protein immunogen on the OMP of 24% clinical isolates of *P. multocida*. This MAb protected mice, rabbit with passive immunization against challenge (37). The major 38 kDa protein of OMP recognized by immune sera was afforded to be vaccine candidate by many reports (35, 86, 105, 120).

The reactions of LPS extract of Pm-vac and 2T35 were observed at 7 kDa by various types of rabbit immune sera. LPS extract of Pm-vac was recognized by immune sera against sonicated cell Pm-vac, 3005 and KSCN antigen extract, but LPS extract of 2T35 was recognized by immune sera against sonicated cell Pm-vac, 2T35 and capsule extract 2T35.

The antigen extract of *P. multocida* Pm-vac parental strain and 2T35 mutant strain were recognized by most immune sera with strong and weak reaction on various protein components, normal rabbit sera showed no reaction with protein antigens. Some factors such as heat during transfer of antigen to nitrocellulose may be the cause of denaturation and change in antigenic determinants of protein antigens, and weak reaction with immune sera were observed.