A 39-kDa Capsular Protein is a Major Cross-Protection Factor as Demonstrated by Protection of Chickens with a Live Attenuated *Pasteurella multocida* Strain of P-1059

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ABSTRACT. The aim of this study was to show that a 39-kDa protein or OmpH of *Pasteurella multocida* strain P-1059 is essential for cross protection. Strain PBA322, a thinly capsulated strain of *P. multocida* strain P-1059, was used as a live vaccine in chickens. Strain PBA322 is a thinly capsulated strain in comparison with the parental strain P-1059. Chickens were vaccinated by single injection and then challenge-exposed with strains P-1059 or X-73 at two weeks post vaccination. Moreover, immune responses were also evaluated for both humoral and cellular immune response by ELISA and lymphocyte proliferation assay, respectively. The results showed that the live vaccine induced efficient immunity to protect chickens from challenge-exposure to the parent strain, but that the heterologous protection was poor. We concluded that the 39-kDa protein is essential for cross protection.

KEY WORDS: 39-kDa protein, cross protection, fowl cholera, *Pasteurella multocida*.

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Pasteurella multocida is a gram-negative bacterium and the causative agent of fowl cholera, bovine or buffalo hemorrhagic septicemia and swine atrophic rhinitis. The bacteria can be classified into 5 capsular serogroups, A, B, D, E and F [20], and 16 somatic serotypes, 1–16 [11]. Strains of capsular serogroup A and somatic serotypes 1, 3 and 4 are known as the causative agents of fowl cholera [20–22]. The virulence factors of *P. multocida* including capsular protein have been demonstrated [2–5, 7–9, 11, 14, 16, 17, 19]. The bacterial capsule plays a role in adherence to epithelial cells of the host at the early stage of infection [10, 14]. The 39-kDa protein in crude capsular extract (CCE) of strain P-1059 (serovar A:3) is an adhesive protein and located in the bacterial capsule [2, 5]. N-terminal sequence analysis of this protein showed that it was identical to the major outer membrane protein H (OmpH) of strain P-1059 [6]. The mutant strain PBA322 of *P. multocida* strain P-1059 was constructed by inhibition of this 39-kDa protein synthesis [23]. Moreover, although a high number of bacteria were inoculated, loss of capsule synthesis was attenuated in the mutant strain, and the protection conferred in the chicken against challenge-exposure with the predominant strains of fowl cholera needs to be clarified. Therefore, the aim of this study was to show that a 39-kDa protein or OmpH of strain P-1059 is essential for cross protection.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions: The bacterial strains used in this study were *P. multocida* strains X-73 (serovar A:1), P-1059 (serovar A:3) and PBA322 (serovar A:3) [6, 23], respectively. Strain PBA322, a thinly capsulated strain, was constructed by inhibition of 39-kDa protein synthesis as described in a previous study [23]. *P. multocida* strains were grown in brain heart infusion (BHI; Becton, Dickinson and Co., Sparks, MD, U.S.A.) broth at 37°C for 6 hr and then subcultured on dextrose starch agar (DSA; Becton, Dickinson and Co.) at 37°C for another 18 hr before being used in the experiments.

Vaccine: Strain PBA322 from strain P-1059 was used as a live attenuated vaccine in this study. One single colony of strain PBA322 was inoculated in BHI broth and incubated at 37°C until the OD₆₀₀ reached 0.1. In order to enumerate the viable bacterial number, a serial tenfold dilution was performed in BHI broth before spreading on a DSA plate. Moreover, a sterile BHI broth was used as a negative control vaccines.

Chickens: A total of 40 eight-week-old *P. multocida*-antibody-free layers (Hi-Sex; RPM Farm & Feed Co, Ltd., Chiang Mai, Thailand) were used in this study (Table 1). Three milliliters of blood was collected from all the chickens before each vaccination or challenge-exposure in order to evaluate the immune responses by enzyme-linked immunosorbent assay (ELISA) and lymphocyte proliferation assay (LPA). The use of laboratory animals was reviewed by the animal welfare committee of the Faculty of Veterinary Medicine, Chiang Mai University. Experiments were performed in a closed system. Experiment rooms and instruments were cleaned with disinfectant for two weeks before and after the

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Table 1. Experiments and results

Group of chickens	Vaccine ^{a)}	No. of chickens	Challenge ^{b)}	No. survivors / No. vaccinations (% of survivors)
1	PBA322	10	P-1059	10/10 (100)
2	Sterile BHI broth	10	P-1059	0/10 (0)
3	PBA322	10	X-73	0/10 (0)
4	Sterile BHI broth	10	X-73	0/10 (0)

a) Strain PBA322 was used as a live vaccine for groups 1 and 3. Bacteria were grown in BHI broth at 37°C until the OD₆₀₀ reached 0.1. Then, the concentration was adjusted to 3.3×10^8 cfu/ml of bacteria number. All vaccines were introduced to chickens by intramuscular administration into pectoral muscle. Sterile BHI broth was injected into chickens in groups 2 and 4 as negative control vaccines.

b) Strains of *P. multocida* were intramuscularly administered into pectoral muscle at challenge. The number of bacteria per challenge-exposure was 2.3×10^3 (X-73) or 4.3×10^4 (P-1059) cfu/ml, respectively.

experiment. Waste products were treated before release into the environment.

Vaccinations: Chicken were divided into 4 groups (groups 1–4) based on the strains for challenge-exposure (Table 1). Groups 1 and 3 were vaccinated with a live vaccine as experiment groups, while groups 2 and 4 were vaccinated with a sterile BHI broth as negative groups. Chickens of groups 1 and 3 were vaccinated with a single dose of a live vaccine at a concentration of 3.3×10^8 cfu/ml.

Determination of humoral immune response: Antibody titers were determined by the indirect ELISA [24]. MicroWell plates (Nunc-Immuno™ plates, Denmark) were coated with 0.3% formalinized whole cells of *P. multocida* strains X-73 or P-1059 in coating buffer, 0.5 M carbonate buffer (pH 9.6) and incubated at 4°C overnight. Plates were incubated with blocking buffer (1% skim milk in PBS) for 1 hr to prevent nonspecific binding. Each chicken serum diluted with blocking buffer was then added into each well of the plate. After washing the plates three times with PBS-T, a 1:1,000 dilution of horseradish peroxidase-conjugated anti-chicken IgY (IgG; Sigma Aldrich, St. Louis, MO, U.S.A.) in blocking buffer was added to each well. Substrate solution (30 mg o-phenylenediamine dihydrochloride, O-PDA; Wako, Osaka, Japan) in substrate buffer (0.2 M Na₂HPO₄·12H₂O and 0.1 M C₆H₈O₇; pH 4.8) was added, and the reaction was stopped by adding 2 N sulfuric acid (H₂SO₄; Wako). Then, the absorbance values were recorded at a wavelength of 492 nm using an ELISA reader (Immuno Mini NJ-2300, Intermed, Osaka, Japan). Results were expressed as log antibody titers calculated as follows:

$$\text{Log antibody titer} = (1.464 \times \log_{10} \text{Sp value}) + 3.197$$

Abs.=absorbance

$$\text{Sp value} = [(\text{Abs. sample}) - (\text{Abs. negative control})] / (\text{corrected positive control})$$

$$\text{Corrected positive control} = (\text{Abs. positive control}) - (\text{Abs. negative control})$$

The average antibody titer of each group was calculated as the standard mean, and the calculated values were used to

compare the statistical data.

Determination of cellular immune response: An *in vitro* LPA was adapted and performed as described previously [13, 18]. Peripheral blood mononuclear cells (PBMCs) were prepared from three milliliters of blood by a gradient centrifugation technique [10] with Ficoll® gradient (Amersham Pharmacia Biotech, Uppsala, Sweden) and centrifuged at 400 g for 30 min. PBMC fraction was collected and washed twice with sterile RPMI tissue culture medium (RPMI1640, 31800-022, Gibco, Invitrogen, Carlsbad, CA, U.S.A.) supplemented with 100 IU/ml streptomycin and 100 IU/ml penicillin (RPMI). Subsequently, pellets were resuspended with 4 ml R10 culture medium [RPMI tissue culture medium supplemented with 100 IU/ml streptomycin, 100 IU/ml penicillin, 10% fetal calf serum (FCS, 10270-098, Gibco) and 2.5×10^{-5} M 2-mercaptoethanol], before enumerating the number of cells. PBMCs at 1×10^6 cells/well were stimulated in triplicate with 5.0 µg/ml (final concentration) of crude capsular antigen (CCE) from strains X-73 and P-1059 in a 96-well U-bottom microtiter plate. R10 culture medium and 10 µg/ml of ConA (Concanavalin A, C-2010, Sigma) were used as a cell control and mitogen control, respectively. Then the microtiter plate was incubated at 42°C for 48 hr in a humidified atmosphere with 5% CO₂. In the last 16 hr before harvesting, cultures were pulsed with 0.25 µCi of methyl-[³H]-thymidine. Thymidine uptake was determined with a liquid scintillation counter (MicroBeta TriLux, Wallac, Finland). Results were expressed as stimulation indices (SI), calculated as SI=mean cpm in stimulated wells/mean cpm in media wells.

Protection assay: Chickens were challenge-exposed at two weeks post vaccination. Chickens of groups 1 and 2 were challenge-exposed with 4.3×10^4 cfu/ml of the parent strain P-1059 to determine the homologous protection, while groups 3 and 4 were challenge-exposed with 2.3×10^3 cfu/ml of strain X-73 to determine the heterologous protection [24]. The birds were observed for their mortality rates and clinical signs for 10 days.

Statistical analyses: Comparisons of protection in immunized chickens were made using Fisher's exact test.

Fig. 1. Antibody responses in the sera of chicken to *P. multocida* strain P-1059. ELISA plates were coated with whole cells of *P. multocida* strain P-1059. Chickens were divided into 4 groups (groups 1–4) based on vaccine types (Table 1). The data are presented as the log mean endpoint titers, and the bars indicate standard errors of the means.

Fig. 2. Antibody responses in the sera of chicken to *P. multocida* strain X-73. ELISA plates were coated with whole cells of *P. multocida* strain X-73. Chickens were divided into 4 groups (groups 1–4) based on vaccine types (Table 1). The data are presented as the log mean endpoint titers, and the bars indicate standard errors of the means.

RESULTS

Experiment in chickens: Groups of chickens, vaccines and results are shown in Table 1. During ten days post challenge-exposure, all chickens of groups 2 and 4 were found dead at 12–24 hr post exposure. Necropsies were performed, and samples were collected and sent to confirm cause of death at a microbiological laboratory. The carcasses showed the typical gross lesions of fowl cholera, e.g., multiple necrotic foci in the liver and/or spleen, lung congestion and edema, multiple petechiae in liver, hemorrhage in the small intestines and splenomegaly. Chickens in the experimental groups began to show clinical signs, for example, depression and loss of appetite, at 6 to 8 hr after exposure. Then, at 12 hr after

exposure, chickens started to die, and dead chickens were found until three days after the exposure. Bacterial isolation showed pure colonies on the agar plates, and biochemical reactions of the isolates showed the typical properties of *P. multocida* (data not shown).

Protection assay: Chickens in group 1 were challenge-exposed with the parent strain of the vaccine, strain P-1059, and complete protection (100% survived) was obtained. In contrast, no survivors (0%) were observed in group 3, which were challenge-exposed with strain X-73. Fisher's exact test indicated that there was significant difference in cross-protection conferred by the live vaccine ($P < 0.05$).

ELISA: The average log antibody titers of chicken antiserum against strains P-1059 and X-73 are shown in Figs. 1, 2.

Fig. 3. Lymphocyte proliferation assay for strain P-1059.

Lymphocytes of chickens in each group were collected and cultured in the presence of ConA or cell lysate antigens of strain P-1059 (A:3) at $5 \mu\text{g/ml}$ for 48 hr. Cell proliferation was measured by measuring $[^3\text{H}]$ thymidine uptake after 48 hr of culture. The data are presented as the mean stimulation index (SI), and the bars indicate standard errors of the means. The stimulation index (SI) was calculated by the following formula: $\text{SI} = (\text{mean cpm of mitogen- or antigen-stimulated proliferation}) / (\text{mean cpm of non-stimulated proliferation})$.

Fig. 4. Lymphocyte proliferation for against strain X-73.

Lymphocytes of chicken in each group were collected and cultured in the presence of ConA or cell lysate antigens of strain X-73 (A:1) at $5 \mu\text{g/ml}$ for 48 hr. Cell proliferation was measured by measuring $[^3\text{H}]$ thymidine uptake after 48 hr of culture. The data are presented as the mean stimulation index (SI), and the bars indicate standard errors of the means. The stimulation index (SI) was calculated by the following formula: $\text{SI} = (\text{mean cpm of mitogen- or antigen-stimulated proliferation}) / (\text{mean cpm of non-stimulated proliferation})$.

The results showed that strong humoral immune responses were generated by vaccination against homologous strain P-1059 (Fig. 1), while there was no response against heterologous strain X-73 (Fig. 2). The chicken antibody titers of all the groups before vaccination against both strains were not significantly different. Antibody titers against homologous strain P-1059 were empirically elevated in the vaccinated chickens (groups 1 and 3) post vaccination, while the non-vaccinated chickens' immune responses (groups 2 and 4) were not elevated. The increased antibody titers post vaccination against homologous strain P-1059 of the two vaccinated groups (groups 1 and 3) were not significantly different. Moreover, the antibody response of the vaccinated groups against homologous strain P-1059 were significantly different from those of the non-vaccinated groups ($P < 0.05$). In contrast, there was no antibody response against heterolo-

gous strain X-73 in the period spanning from before vaccination right up until after vaccination (Fig. 2).

LPA: The LPA data are shown in Figs. 3, 4. The SI values for the pre-vaccination groups were not significantly different even though when stimulated with antigens of *P. multocida* strains P-1059 or X-73. After vaccination, lymphocytes of the vaccinated chickens slightly proliferated when compared to lymphocytes of the non-vaccinated groups when stimulated with strain P-1059 (Fig. 3). The SI values of the vaccinated groups were significantly different from SI values of the non-vaccinated groups, even though when stimulated with *P. multocida* strains P-1059 ($P < 0.05$). In contrast, the SI values of lymphocytes stimulated with strain X-73 were not different among the vaccinated or non-vaccinated chickens (Fig. 4).

DISCUSSION

Cross protection might be affected by lack of the 39-kDa protein in the mutant PBA322. Previous investigations indicated that both native and recombinant 39-kDa proteins are cross-protective adhesive antigens of *P. multocida* capsular serogroup A strains [4, 24]. Therefore, existence of the 39-kDa protein in bacterial capsule corresponds to a bacterial cross-protective immunogen. As expected, a low level of protection was conferred by the heterologous challenge-exposure. Previous studies constructed and determined the protection conferred by mutants of *P. multocida* [12, 15]. The acapsular *P. multocida* strain PBA930, a *hexA::tet* (*M*) mutant, was able to protect chickens against challenge with wild-type strain X-73 [12]. Moreover, the attenuated *aroA* mutant PMP3 conferred efficient protection against challenge with strains X-73 and P-1662 [15]. Therefore, the present protection assays indicate that the inhibition of 39-kDa protein synthesis causes a thin encapsulated strain, but the strain may lack a cross-protective characteristic.

Live noncapsulated mutants were attenuated and also proved to be efficacious live vaccine candidates for mice and natural hosts [12, 15]. Theoretically, a live vaccine activates all phases of the immune system. It elicits a humoral IgG and local IgA, raises an immune response to all protective antigens, offers more durable immunity and is more cross-reactive. In the present study, the antibody titer profiles showed low antibody response in strain X-73-coated ELISA, but in contrast, a very high response was seen in strain P-1059-coated ELISA. This correlated to the characteristic of the strain. In our previous work, strain PBA322 was constructed by inhibition of this cross-protective antigen, a cross-protective antigen among *P. multocida* capsular type A strains [23], and subsequently, a cross-protective characteristic of the strain must be deleted. Therefore, the antibody induced by vaccination with strain PBA322 reacted particularly to a homologous strain. The LPA results indicated that the lymphocytes of chickens vaccinated with the mutant were not correlated with the protection. In accordance with the LPA profiles, chicken lymphocytes showed low proliferation after vaccinations, even when cells were stimulated with homologous strain P-1059. However, the mechanism of cellular immune response to the present live attenuated strain needs to be clarified. The role of the cellular immune response in the protection is still questioned, even though the bacterium has been demonstrated to be an intracellular bacterium [1, 16]. A previous study suggested that there is no evidence of a major role of cell-mediated immunity induced by *P. multocida* vaccine for hemorrhagic septicemia, because *P. multocida* has not been described as an intracellular pathogen [25]. In contrast, Ibrahim *et al.* [16] and Al-haj Ali *et al.* [1] characterized the ability of *P. multocida* to penetrate host cells, and their results suggested that *P. multocida* might be an intracellular pathogen. Ibrahim *et al.* [16] reported that *P. multocida* could multiply intracellularly in hepatocytes or heart tissue of a host and employ the glycoprotein as an invasive factor for receptors on the chicken embryo fibroblast cell surface [1]. However, the intracellular multiplication

and cellular immune response of the bacterium need to be clarified.

In conclusion, the cross protection conferred by vaccination with a live vaccine, a 39-kDa protein knockout strain, was poor, and the results showed that a 39-kDa protein of *P. multocida* is essential for the cross protection. Moreover, the cross protection is mainly the result of humoral immunity even though the bacterium is able to multiply intracellularly.

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