Iranian Journal of Veterinary Research, Shiraz University, 2013, Vol. 14, No. 3, Pages 197-202

Immune efficacy of OmpH and OmpA DNA vaccines against avian *Pasteurella multocida*

Gong, Q.[1*]; Qin, C. L.[1]; Niu, M. F.[1]; Cheng, M.[2]; Sun, X. F.[2] and Zhang, A. G.[2]

[1]Department of Biological Engineering, School of Food and Biological Engineering, He Nan University of Science and Technology, Luoyang, 471003 PR, China; [2]MSc in Molecular Biology, Department of Biological Engineering, School of Food and Biological Engineering, He Nan University of Science and Technology, Luoyang, 471003 PR, China

[*]**Correspondence:** Q. Gong, Department of Biological Engineering, School of Food and Biological Engineering, He Nan University of Science and Technology, Luoyang, 471003 PR, China. E-mail: gongqiang79@126.com

(Received 2 Apr 2012; revised version 27 Oct 2012; accepted 21 Jan 2013)

Summary

Avian *Pasteurella multocida* is an agent of fowl cholera. The protective effect achieved through orthodox vaccines is not ideal. The research on novel vaccines against avian *Pasteurella multocida* is imperative. In this study, the genes encoding outer membrane protein H and A (OmpH and OmpA) were cloned into the eukaryotic expression vector pcDNA$^{3.1(+)}$ and the recombinant plasmids, namely DNA vaccines (pOMPH and pOMPA) were obtained. Five groups of chickens (n=20 per group) were intramuscularly injected with the two recombinant plasmids, attenuated live vaccine, control vector pcDNA$^{3.1(+)}$ and PBS, respectively. The immune responses and protective efficacy were evaluated after immunization by serological and challenging. A significant increase in serum antibody levels was observed in chickens vaccinated with the attenuated live vaccine and the two DNA vaccines. Additionally, the lymphocyte proliferation (SI values) were higher in chickens immunized with the attenuated live vaccine and the two DNA vaccines than in those vaccinated with pcDNA$^{3.1(+)}$ and PBS ($P<0.05$). Furthermore, the two DNA vaccines provided partial protection to the vaccinated chickens; however, the protective efficacy was inferior to that provided by the attenuated live vaccine.

Key words: Avian *Pasteurella multocida*, pOMPH, pOMPA, DNA vaccine, Immune efficacy

Introduction

Fowl cholera is a serious infectious disease of poultry and is widely distributed in many countries. Currently, the vaccines for prevention of fowl cholera include attenuated vaccine and inactivated vaccine. The former is the most widely studied in the field of poultry attenuated vaccine researched in China and peaked in the 1980's. Nevertheless, the antigenic structure of avian *Pasteurella multocida* is complicated, and prone to mutation (Davies *et al*., 2003). The protective effect achieved through attenuated live vaccine is not ideal and the immunological effect of inactivated vaccine is not as good as live vaccine. Thus, development of new generation vaccine systems to prevent fowl cholera is important to avoid the disadvantages of the currently used vaccines (Daboa *et al*., 2008).

Since it was successfully prepared by Wollf in the 1990's, DNA vaccine, which has the advantages of convenient preparation, low cost, and is easy to preserve, is currently the subject of intense investigation in the field of vaccine research (Bonato *et al*., 1998). Data from many studies have provided proof of the principle that DNA vaccines are effective at inducing both humoral and T cell responses (Oveissi *et al*., 2009). It can provide prolonged antigen expression, leading to boosting immune response and inducing memory response against infection. Furthermore, the endogenous expression of antigen from DNA vaccine introduced into host cells leads to the processed peptide with the

MHCl being able to induce CTL. The induction of potent cellular immune responses often gives DNA vaccination an immunological advantage over subunit protein vaccination (Gurunathan *et al*., 2000; Smooker *et al*., 2004). However, the researches on DNA vaccine against *P. multocida* have been less reported, in particular fowl cholera. The studies of novel vaccines on these kinds of diseases are mainly in genetic engineering subunit vaccine, especially swine lung plague (Liao *et al*., 2006; Hsuan *et al*., 2009). In this study, we used the protective antigen genes of *ompH* and *ompA* avian *P. multocida* as the basis for construction of two DNA vaccines. The chickens were immunized with the two DNA vaccines and challenged by avian *P. multocida*. Then the immune efficacy were detected. The purpose of this study was to lay a foundation for further research and development of vaccines against avian *P. multocida* infection.

Materials and Methods

Construction of plasmids

Genomic DNA of the avian *P. multocida*-CVCC474 strain (serotype A:1) was extracted according to the conventional method. The *ompH* and *ompA* gene fragments were amplified using the genomic DNA as a template. Primers were designed according to the nucleotide sequences of the *ompH* and *ompA* genes of *P. multocida* strain Pm-17 (GenBank accession number DQ417897.1) and 95010872 (GenBank accession number AY643794.1), respectively. Table 1 lists the primer sequences. The amplified products were purified using a gel extraction mini kit and were ligated into the eukaryotic expression vector pcDNA$^{3.1(+)}$ (Invitrogen, San Diego, CA, USA), resulting in two DNA vaccines namely recombinant pasmid pcDNA-OMPH (pOMPH) and pcDNA-OMPA (pOMPA). Then the plasmids were properly verified by sequencing.

In vitro expression of recombinant plasmids

One day before transfection, mouse myeloma Sp2/0 cells at logarithmic growth phase were seeded into 24-well plates (Greiner Bio-One, longwood, Germany) and grown at 37°C, 5% CO_2 until 80% confluency. Plasmid pOMPH, pOMPA and pcDNA$^{3.1(+)}$ were extracted using a commercial plasmid mini kit (Yuan Ye Biotech Co., Shanghai, China). Then the Sp2/0 cells were transfected with 0.8 µg of pOMPH, pOMPA and empty vector pcDNA$^{3.1(+)}$ using 2 µl of Lipofectamine 2000 (Gibco, Grand Island, NJ, USA) followed by indirect immunofluorescent testing after 72 h. Briefly, the cells were fixed with methanol/acetone for 20 min at room temperature, followed by addition of 200 µl rabbit anti-avian *Pasteurella multocida* Omps polyclonal antibody (1:40 dilution), and incubated in a moist chamber for 1 h at 37°C. Next, 200 µl fluorescein isothiocyanate (FITC)-labelled goat anti-rabbit IgG (1:100 dilution, Sigma, St. Louis, MO, USA) containing 1% Evans blue was added; the samples were incubated in a moist chamber for 1 h at 37°C, and were then observed in a drop of basic glycerine under an inverted microscope to visualize blue-green fluorescence in Sp2/0 cells.

Vaccination protocol

None of the immune chickens (n=100), 1-day-old, were randomly assigned to immunization groups containing 20 chickens each. DNA vaccination was carried out when the chickens were bred to 4 weeks of age. The plasmids were prepared on a large scale using the alkaline lysis method and were purified with a endo-free plasmid maxi kit (Omega, USA). The plasmid preparations were then adjusted to 1 µg/µl using

Table 1: Primers sequences of o*mpH* and *ompA* gene

Names	Oligonucleotides primer sequences	Reference (GenBank accession number)
ompH forward	5′- TGAGGTACCATGAAAAAGACAATCGTAG-3′	(AF154834.1)
ompH reverse	5′- TAGGAATTCTTAGAAGTGTACGCGTAAAC -3′	(AF154834.1)
ompA forward	5′-GCGGTACCATGAAAAAAACAGCAATTGC-3′	(AF154834.1)
ompA reverse	5′-GCGGGATCCTTATTTGTTACCTTTAACAGCG-3′	(AF154834.1)

phosphate-buffered saline (PBS: 0.01 M, pH = 7.2). The chickens were immunized with 200 µg of pOMPH, pOMPA, pcDNA$^{3.1(+)}$ and 200 µl PBS three times at 2-week intervals by intramuscular injection for DNA immunization groups and the negative control groups. In the positive control group, chickens were intramuscularly injected with 0.5 ml attenuated live vaccine of avian *P. multocida* at the time of initial vaccination.

ELISA

After immunization, blood samples were drawn from the chickens and serum specimens were separated. The serum antibody titers were tested using indirect enzyme-linked immunosorbent assay (ELISA). The coating antigen was avian *P. multocida* suspension (10^9 cfu/mL) and the anti-antibody was rabbit anti-chicken IgG-Horseradish peroxidase. Absorption was measured at 492 nm.

Lymphocyte proliferation assay

Firstly, avian *P. multocida* Omps were extracted by supersonic split (Hu *et al*., 2007). Two weeks after each immunization, blood from the chickens in each group was collected. The peripheral blood lymphocytes (PBL) were separated by density gradient centrifugation. After collection of the cells under sterile conditions, they were resuspended to a concentration of 1×10^7 cells/ml and added to 96-well plate. Each well was pulsed with 50 µl of 20 µg/ml avian *P. multocida* Omps (experimental well) or 50 µl RPM1640 medium (negative control). After 60 h cultivation, 10 µl of 5 mg/ml MTT was added to each culture well and incubated for 3 h. After being centrifuged for 10 min, the supernatant was discarded and 150 µl of dimethyl sulfoxide (DMSO) was added to the pellet and incubated for 10 min until crystals dissolved. The OD value of each well was measured with a test wavelength of 570 nm and the SI values were determined from the formula:

SI=OD (experimental well)/OD (negative control well) (Gupta *et al*., 2007).

Challenge

There are several different challenge models available, such as intravenous, intraperitoneal and intramuscular. In this study, the chickens were challenged 2 weeks after 3rd DNA immunization with the $5LD_{50}$ (2.5×10^4 CFU) virulent *P. multocida* strain CVCC474 by intramuscular rout. The chickens were reared for two weeks, the survival number and protection rate were counted.

Results

In order to investigate the expression of plasmids *in vitro*, the indirect immuno-fluorescence was down. As shown in Figs. 1A, B and C, sp2/0 cells transfected with plasmids pOMPH, and pOMPA showed homogeneous blue-green fluorescence under an inverting microscope, demonstrating that avian *P. multocida* genes, *ompH* and *ompA* were transfected *in vitro* into Sp2/0 cells and that the cells expressed the target proteins.

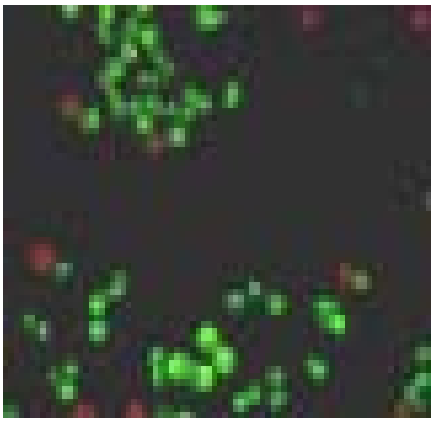
After vaccination, the serum antibody titers were tested using indirect ELISA. As shown in Fig. 2, after immunization, the serum antibody levels in the pOMPH-, pOMPA- and attenuated live vaccine-groups were significantly higher than those in the negative control groups ($P<0.01$). From 4 weeks after the first vaccination, the serum antibody levels in attenuated live vaccine-group were higher than those in pOMPH group and pOMPA group ($P<0.05$). Although the serum antibody levels in the pOMPH group were slightly higher than that in pOMPA group at the 4 and 6 week point, there is no apparent difference between them ($P>0.05$).

To investigate the cellular immune response induced by the two DNA vaccines, the proliferation of peripheral blood lymphocytes at three time points after vaccination during each experiment were detected. In each experiment, the SI values for the two DNA vaccine-groups and attenuated live vaccine-group were consistently higher than those for the negative control groups ($P<0.05$). However, there is no difference in the values among the groups of attenuated live vaccine and DNA vaccines ($P>0.05$) (Fig. 3).

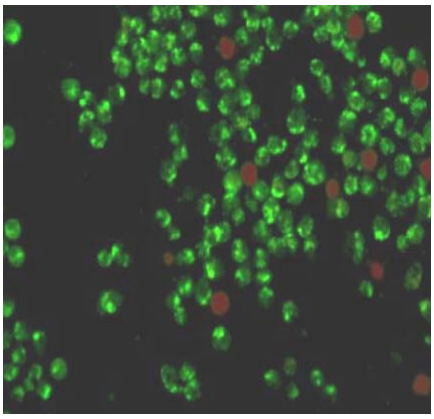
Groups of chickens were challenged with virulent avian *P. multocida* CVCC474 strain 2 weeks after last immunization.

Survival number and protection rate were counted till 14 days (Table 2). The result showed that the number of death in pcDNA$^{3.1(+)}$ and PBS groups was higher than the two DNA vaccines and the attenuated live vaccine. However, the survival of chickens immunized with pOMPH (60%) and pOMPA (50%) were lower than those in the chickens immunized with the attenuated live vaccine (75%) ($P<0.05$).

A



B



C

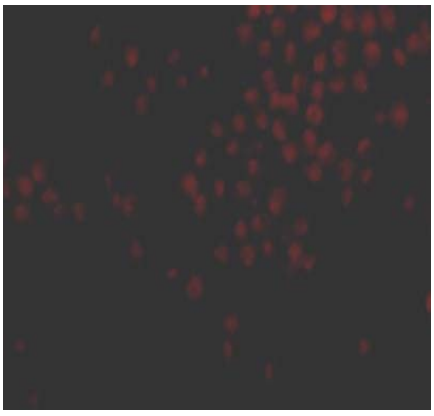


Fig. 1: Immunofluorescence in Sp2/0 cells transfected with pOMPH (A), pOMPA (B) and vector pcDNA$^{3.1(+)}$ (C)

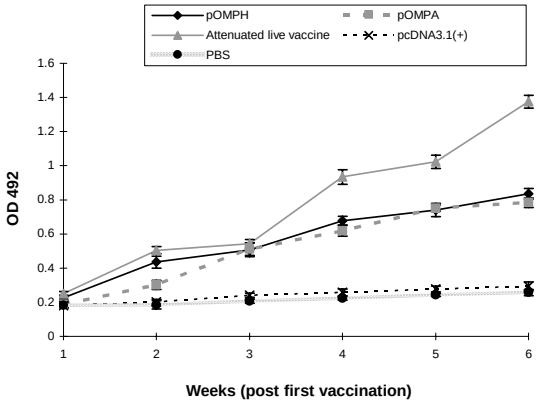


Fig. 2: Dynamic changes of serum antibodies in immune chickens. Following the first immunization, serum antibody levels were measured by indirected ELISA weekly until 6 weeks. Chickens were immunized with pOMPH vaccine (◆), pOMPA vaccine (■), attenuated live vaccine (▲), pcDNA$^{3.1(+)}$ vector (×), and PBS (●)

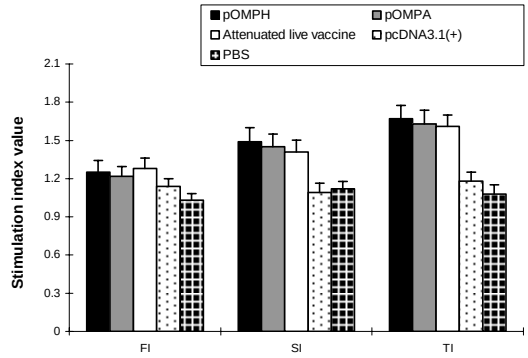


Fig. 3: Proliferation of lymphocytes from chickens immunized with DNA vaccines. The Omps of avian *P. muhocida* was administered to stimulate peripheral blood lymphocytes 2 weeks after each immunization. FI: First immunization, SI: Second immunization, and TI: Third immunization

Table 2: Protection of immunized chickens against lethal challenge with avian *P. multocida*

Groups	Survival number/total	Protection rate (%)
pOMPH	12/20	60*
pOMPA	10/20	50*
Attenuated live vaccine	15/20	75**
pcDNA$^{3.1(+)}$	1/20	5
PBS	0/20	0

* $P<0.05$ and ** $P<0.01$

Discussion

Outer membrane proteins are the mainly immunodominant antigens of *P. multocida*

(Mitchison *et al*., 2000; Prado *et al*., 2005; Hatfaludi *et al*., 2010). OmpH is the super-micropore-protein of *P. multocida*. Luo *et al*.'s study (1997) showed that purified native OmpH was able to confer protection of chickens from fowl cholera by challenge-exposure with virulent strain and the level of protection was equivalent to that achieved with attenuated vaccine. Sthitmatee *et al*.'s research (2008) indicated that OmpH proteins from different serotypes were highly homologous and had cross-protection. Other studies also demonstrated that OmpH could induce high-level immune response to homologous bacteria (Antony *et al*., 2006; Bosch *et al*., 2001; Li *et al*., 2007). OmpA is also one of the cross-protective antigens and arch-outer membrane proteins from *P. multocida*. Lu *et al*.'s experiment (1999) demonstrated that monoclonal antibody of OmpA could degrade the mortality of the experimental animal. However, results of some researches showed that the protective effects provided by OmpA protein were not ideal, although it could induce high level immune response (Gatto *et al*., 2002). Therefore, the immunological effect created by OmpA protein of *P. multocida* is not yet conclusive.

Humoral immune response is an important factor in resistance to avian *P. multocida* infection. In this study, the ELISA experiment was carried out to detect the levels of antibodies induced by the DNA vaccines (pOMPH and pOMPA). We found that the abilities to stimulate humoral immune response of the two DNA vaccines were inferior to that of attenuated live vaccine. The cellullar immunologic response also plays an influential role in the process of anti-infection (Register *et al*., 2007). Lymphocyte proliferation assay is a commonly used method to detect cellular immune function. The stimulator we used was avian *P. multocida* Omps in the lymphocyte proliferation assay. Similar SI values were observed among attenuated live vaccine-group and the two DNA vaccine-groups. However, the protective efficacy conferred by pOMPH and pOMPA vaccines was not as good as the attenuated live vaccine. We hope the results of this study could lay the foundation for the development of novel DNA vaccines against fowl cholera.

Acknowledgement

The authors gratefully acknowledge the grant from the natural scientific research project of Henan Province Education Department, China (No. 12A180009).

References

Antony, PX; Nair, GK; Jayaprakasan, V; Mini, M and George, S (2006). A simple protocol for amplification of genes from inactivated oil adjuvant fowl cholera vaccine. Int. J. Poul. Sci., 5: 623-626.

Bonato, VLD; Lima, VMF; Tascon, RE; Lowrie, DB and Silva, C (1998). Identification and characterization of protective T cells in hsp65 DNA-vaccinated and *Mycobacterium tuberculosis* infected mice. Infect. Immun., 66: 169-175.

Bosch, M; Tarragó, R; Garrido, ME; Campoy, S; Fernández de Henestrosa, AR; Pérez de Rozas, AM, Badiola, I and Barbé, J (2001). Expression of the *Pasteurella multocida* ompH gene is negatively regulated by the Fur protein. FEMS. Microbiol. Lett., 203: 35-40.

Davies, RL; MacCorquodale, R and Caffrey, B (2003). Diversity of avian *Pasteurella multocida* strainsbased on capsular PCR typing and ariation of the OmpA and OmpH outer membrane proteins. Vet. Microbiol., 91: 169-182.

Daboa, SM; Confera, A; Montelongoa, M; Yorka, P and Wyckoff III, JH (2008). Vaccination with *Pasteurella multocida* recombinant OmpA induces strong but non-protective and deleterious Th2-type immune response in mice. Vaccine. 26: 4345-4351.

Gatto, NT; Dabo, SM; Hancock, RE and Confer, AW (2002). Characterization of, and immune responses of mice to, the purified OmpA-equivalent outer membrane protein of *Pasteurella multocida* serotype A:3 (Omp28). Vet. Microbiol., 87: 221-235.

Gupta, VK; Rout, PK and Vihan, VS (2007). Induction of immune response in mice with a DNA vaccine encoding outer membrane protein (omp31) of *Brucella melitensis* 16M. Res. Vet. Sci., 82: 305-313.

Gurunathan, S; Wu, CY; Freidag, BL and Seder, RA (2000). DNA vaccines: a key for inducing long-term cellular immunity. Curr. Opin. Immunol., 12: 442-447.

Hatfaludi, T; Al-Hasani, K; Boyce, JD and Adler, B (2010). Outer membrane proteins of

Pasteurella multocida. Vet. Microbiol., 144: 1-17.

Hsuan, SL; Liao, CM; Huang, C; Winton, JR; Chen, ZW; Lee, WC; Liao, JW; Chen, TH; Chiou, CJ; Yeh, KS and Chien, MS (2009). Efficacy of a novel *Pasteurella multocida* vaccine against progressive atrophic rhinitis of swine. Vaccine. 27: 2923-2929.

Hu, XN; Zhu, RL; Liu, HZ; Bi, JM; Wang, W and Li, XC (2007). Study on extraction and antigenicity of the out-mebrane protein of *Bordetella avium*. Acta Microbiol. Sin., 47: 714-717.

Li, LJ; Li, L; Li, J; Zhang, SH; Wu, B; Chen, HC and Zhou, R (2007). Cloning and characterization of outer membrane protein H of toxigenic *Pasteurella multocida* strain HN-13 of swine. J. Agr. Biotech., 15: 30-33.

Liao, CM; Huang, CJ; Hsuan, SL; Chen, ZW; Lee, WC; Liu, C; Winton, JR and Chien, MS (2006). Immunogenicity and efficacy of three recombinant subunit *Pasteurella multocida* toxin vaccines against progressive atrophic rhinitis in pigs. Vaccine. 24: 27-35.

Lu, YS; Lai, WC; Pakes, SP and Nie, LC (1999). A monoclonal antibodies against a *Pasteurella multocida* outer membrane protein protects rabbits and mice against Pasteurellosis. Infect. Immun., 59: 172-180.

Luo, Y; Glisson, JR; Jackwood, MW; Hancock, RE; Bains, M; Cheng, IHa and Wang, C (1997). Cloning and characterization of the major outer membrane protein gene (OmpH) of *Pasteurella multocida X-73*. J. Bacteriol., 179: 7856-7864.

Mitchison, M; Liu, W; Kwang, J; Wilkie, I and Adler, B (2000). Overexpression and immunogenicity of the Oma87 outer membrane protein of *Pasteurella multocida*. Vet. Microbiol., 72: 91-96.

Oveissi, S; Omar, AR; Yusoff, K; Jahanshiri, F and Hassan, SS (2009). DNA vaccine encoding avian influenza virus H5 and Esat-6 of *Mycobacterium tuberculosis* improved antibody responses against AIV in chickens. Comp. Immunol. Microb. 8: 326-328.

Prado, ME; Dabo, SM and Confer, AW (2005). Immunogenicity of iron-regulated outer membrane proteins of *Pasteurella multocida* A:3 in cattle: molecular characterization of the immunodominant heme acquisition system receptor (HasR) protein. Vet. Microbiol., 105: 269-280.

Register, KB; Sacco, RE and Brockmeier, SL (2007). Immune response in mice and swine to DNA vaccines derived from the *Pasteurella multocida* toxin gene. Vaccine. 25: 6118-6128.

Smooker, PM; Rainczuk, A; Kennedy, N and Spithill, TW (2004). DNA vaccines and their application against parasites-promise, limitations and potential solutions. Biotechnol. Annu. Rev., 10: 189-236.

Sthitmatee, N; Numee, S; Kawamoto, E; Sasaki, H; Yamashita, K; Takahashi, N; Kataoka, Y and Sawada, T (2008). Protection of chickens from fowl cholera by vaccination with recombinant adhesive protein of *Pasteurella multocida*. Vaccine. 26: 2398-2407.