Outer Membrane Protein A (OmpA) Conferred Immunoprotection against Enterobacteriaceae Infection in Mice

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ABSTRACT

In order to investigate the protective effect of outer membrane protein A (OmpA) against *Enterobacteriaceae* infection in mice, *ompA* gene was cloned from bovine mastitis *E. coli 308-2* isolate, and then the recombinant OmpA protein was expressed and purified. SDS-PAGE detected recombinant OmpA protein and Western blotting confirmed that the protein had an average molecular weight of 60 kDa. Immunological analysis indicated that OmpA protein induced high level of antibodies, IFN-γ and IL-4 cytokines. Moreover, OmpA protein not only conferred a high level of immunogenicity to protect the immunized mice against the challenge of *E. coli*, but also generated protection against *Klebsiella pneumonia* and *Shigella flexneri*. From the data generated during the study it was suggested that OmpA could be selected as a potential candidate for vaccine against *Enterobacteriaceae*.

Key Words: *Enterobacteriaceae*, outer membrane protein A, immunoprotection.

INTRODUCTION

Enterobacteriaceae family is the major worldwide pathogen in the etiology of bovine disease, such as mastitis (1). Among the *Enterobacteriaceae*, *Escherichia coli* (*E. coli*) is an important environmental pathogen in dairy cows (2, 3). Furthermore other *Enterobacteriaceae* such as *Klebsiella pneumonia* (*K. pneumonia*), *Shigella flexneri* (*S. flexneri*) and other bacteria cause bovine diseases (4, 5). In spite of the frequent occurrence of the *Enterobacteriaceae* in the veterinary clinic, an efficient therapy has not yet been established. Traditional control measures including antibiotic therapy and selective culling have serious drawbacks in controlling *Enterobacteriaceae* infection (6). Thus, new vaccine strategies are desirable and therefore the identification of proteins that can elicit protective immunity has become a major focus of current *Enterobacteriaceae* vaccine research.

Outer membrane proteins (Omps) found in Gram-negative bacteria serve as a protective barrier against the external environment, and provide a variety of functions including passive and active transport, host-pathogen recognition, signal transduction, and catalysis (7). Due to their exposed epitopes on the cell surface and highly conserved immunogenicity (8, 9, 10), Omps could be used as candidates to develop vaccines for combating bacterial infections (11, 12). As a most important member of Omps, the outer membrane protein A (OmpA) is a class of highly conserved proteins among the *Enterobacteriaceae* family (7). To date, OmpA is confirmed as a multifunctional protein that plays an important role in bacterial physiology and pathogenesis. It can function as an adhesin and invasin, participate in biofilm formation, act as both an immune target and evasin, and serve as a receptor for several bacteriophages (8, 13).

Some previous reports showed that OmpA activated antigen-presenting cells induced special immunological responses, which suggested that OmpA may be a potential vaccine carrier molecule (7). Huang *et al.* (14) reported protective immunity against *Riemerella anatipestifer* infection in ducks by immunizations with recombinant proteins OmpA. Maiti *et al.* (15) found common carp vaccinated with recombinant OmpA protein elicited high antibody production.

Data regarding the investigation on the immunogenicity of OmpA on *Enterobacteriaceae* family has been very limited. To our knowledge, there has no report on the protection of OmpA against *Enterobacteriaceae* isolated from bovine mastitis. In our study, *ompA* gene of *E. coli* 308-2 from bovine mastitis was cloned and expressed in Rosetta host cells. The OmpA was purified as His_6-tag fusion proteins and screened for immunoprotective efficacy of *Enterobacteriaceae* infection in Balb/c mice.

MATERIAL AND METHODS

Bacterial strains, host cells, plasmids and animals

A total of 4 bacterial strains *308-2*, *2002-1*, *C83919*, and *O157* were isolated from 20 milk samples of clinical bovine mastitis cases from 10 dairy farms (16), and identified as *E. coli* by conventional microbiological methods including gram stain, colony morphology, and coagulase test with rabbit plasma and by ubiquitous DNA-based assays (4). *K. pneumonia* and *E. coli J96* strain was kindly provided by Wei L (Hebei Medical University, China). Standard strain *S. flexneri* was purchased from National Institute for Food and Drug Control, China. Host cells (Rosetta), cloning vector (pMD18-T), and expression vector (pET32a) were purchased form Takara Co. Ltd, Dalian, China.

Four-week-old male BALB/c mice (SPF, 18-22 g) were purchased form Changchun Institute of Biological Products, China. The animals were maintained under standard conditions in the animal house (10,000-grade sterilized environment). The animal protocol of this study was approved by IACUC (Institutional Animal Care and Use Committee) at HeiLongJiang BaYi Agricultural University to comply with Chinese Experiment Animal Law.

Amplification of *ompA* gene

Based on the previously published *ompA* gene sequence (GeneBank accession No: BAA35715) (17), one set of primers was chemically synthesized by Shanghai Sunny Biological Co. Ltd, China. The forward primer was 5'-GGGGAGCTC (*Sac*I) ATGAAAAAGACAGCTATCG, and the reverse primer was 5'-GCCAAGCTT (*Hind*III) TTAAGCCTGCGGCTGAGTT. The underlined nucleotides indicated the restriction site which was added to facilitate cloning. After the bacteria were cultured for 12 h at 37 ˚C, genomic DNA of seven isolates was extracted according to the standard protocol (18). The *ompA* gene was amplified using PCR with LA Taq DNA polymerase (Takara, Dalian. China), and the other reagents were added as outlined by the manufacturer's instructions. The PCR reaction contained genomic DNA 1 µL, forward primer 1 µL, reverse primer 1 µL, dNTP 1 µL, 10×buffer 2 µL, polymerase (Takara, DaLian, China) 0.7 µL, and ddH_2O 13.3 µL with 20 µL of the total volume. Each reaction was preceded by an initial denaturation step at 94 ˚C for 5 min and terminated by a single primer extension step at 72 ˚C for 10 min. All amplified PCR products were detected on 1% agarose gels electrophoresis and stained with ethidium bromide (EB). PCR products were reclaimed and purified using Biospin GeL Extraction Kit (Bioer, Hangzhou, China) according to the manufacture's instruction, then sequenced and analyzed.

The amplified PCR products of *E. coli 308-2* and pMD18-T Vector (Takara, Dalian, China) were digested by *Sac* I and *Hind* III. Target DNA was cloned into pMD18-T in terms of products manual (linking reaction: target DNA 2 µL, pMD18-T Vector 0.7 µL, Solution I 5 µL, and ddH_2O 2.3 µL with 10 µL of the total volume). The positive recombinant plasmid (*ompA*-pMD18-T) was confirmed by DNA sequencing (Shanghai Sunny Biological Co. Ltd, China). The sequences were edited using SequencherTM (Gene Codes, Ann Arbor, MI) and aligned with other known sequences contained in the GenBank, and analyzed in BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) (19). After the confirmed *ompA*-pMD18-T was digested by *Sac* I and *Hind* III, *ompA* was subcloned into pET32a (Takara, Dalian, China). Linking reaction system contained: target gene 2.0 µL, pET32a 6.5 µL, T4 Ligastion buffer 1.0 µL, T4 ligase (Takara, Dalian, China) 0.5 µL. The prokaryotic expression plasmids *ompA*-pET32a was confirmed by PCR detection and double enzyme digestion.

Expression and purification of the OmpA

The Rosetta (Takara, Dalian, China) strain was used as a host

for cloning and expression study. Prior to transformation, a $CaCl_2$ method (18) had been carried out to prepare Rosetta competent cell. The resulting recombinant plasmids (*ompA*-pET32a) were transformed into Rosetta. A positive clone was selected, inoculated into Luria Broth media containing ampicillin (LB/Amp^+) and induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) (Sigma, CA, USA) at 37 °C for 1-4 h. The purification of six-histidine (His_6) tag fusion protein was performed by Ni-NTA His·bind^R Resin purification system (Novagen, CA, USA) according to the product's guideline. Besides, non-recombinant Rosetta cells, the uninduced recombinant clone were used as negative controls.

SDS-PAGE and western blot analysis

Bacterial lysates were subjected to 12% gel of the SDS-PAGE protocol (18) and transferred to nitrocellulose membrane Hybond^{TM}-C (Amersham, Sweden) as described by Towbin *et al*. (20). The primary antibody was mouse anti-OmpA polyclonal antibody (1:1500 dilution, prepared in our laboratory) or mouse anti-His_6 monoclonal antibody (1:2000 dilution, Invitrogen, CA, USA). Horseradish peroxidase-conjugated goat anti-mouse antibodies was used as the second antibody. The protein bands were visualized by 3, 3'-diaminobenzidine (DAB, Zhongshan, Beijing, China) as recommended by the manufacturer.

Immunization, determination of IgG antibody and cytokines

Thirteen four-week-old Balb/c mice (total of twelve groups) were randomly divided into one group with 3 for immunological studies (without challenge) and another with 10 for challenge. Six groups were immunized twice at a 3-week interval by subcutaneous injection of 200 μg recombinant OmpA emulsified with Freund Adjuvant Complete F5881 (Sigma, CA, USA) at ratio of 1:1. Control animals were injected with PBS-F5881 (1:1). Prior to blood sampling, animals were anesthetized by an intraperitoneal injection of pentobarbital sodium (80 μg/g body weight). Animals were bled through the saphenous vein at one-week interval until day 56. Sera were collected and kept at -20°C until use.

Mouse sera were determined for the presence of specific immunoglobulin G (IgG) by enzyme-linked immunosorbnent assay (ELISA) (12). Briefly, recombinant antigen (OmpA) was coated onto a 96-well microtiter plate (Nunc, Denmark) (100 μL/well), and then incubated overnight at 4 °C. The plates were washed with PBST (0.1 M PBS containing 0.05% Tween-20). Next, 100 μL primary antibody was added and incubated for 1 h at 37 °C. The plates were washed with PBST and incubated with 100 μL goat anti-mouse IgG conjugated to horseradish peroxidase (Cappel, Durham, NC) for 30 min at room temperature. The plates were washed again with PBST, and 100 μL of tetramethylbenzidine (TMB) (Invitrogen, CA, USA) was added to each well. Each plate was read at an optical density at 450 nm (Bio-Rad, USA).

For cytokine analysis, after preliminary immunization of 7 days, animals were sacrificed by cervical dislocation, and a suspension of splenocytes was prepared as previously described according to the enzyme-linked immunospot assay (ELISPOT) protocol (21). Spot forming cell (SFC) of interferon-γ (INF-γ) and interleukin-4 (IL-4) secreted by splenocytes were determined by the Mouse INF-γ and IL-4 Precoated ELISPOT Kits (Dalewe, Beijing, China) in accordance with product recommendation.

Lethal challenge

For the challenge study, animals from each group were intraperitoneally challenged with 100 μL lethal dose (1×10^4)/mouse of bacteria on day 14 post booster vaccination. The animals were monitored three times a day. Once the animals had serious clinical signs (moribund), they were euthanized. The animals that survived after challenge were sacrificed one week post challenge. Survival number of animal was recorded up to 7 successive days.

Statistical analysis

The results were presented as arithmetic mean of three replicates ± SE (standard error). The analysis of variance (one-way ANOVA) was performed for evaluating statistical significance of antibody and cytokines, and $P < 0.05$ was considered as statistically significant.

RESULTS

Full-length *ompA* gene was amplified by PCR from genomic DNA extracted from *Enterobacteriaceae*. A fragment of 1041 bp was obtained from *E. coli 308-2*, *2002-1*, *C83919*, *O157*, *J96*, *K. pneumonia*, and *S. flexneri* (Figure 1A). Our previous study confirmed *E. coli 308-2* as highly virulent (16).

Thus, the *ompA* gene of *E. coli 308-2* was directly cloned in pET32a Vector under the control of the T7 promoter, and its nucleotide sequence was determined as described. The obtained products were cut with both *Sac* I and *Hind* III, and two fragments of the expected sizes (5900 bp and 1041 bp) were detected (Figure 1B).

All the recombinant OmpA proteins were expressed as His6-tagged fusion proteins in Rosetta host cells. All these proteins were expressed in soluble form to retain their native conformation and allow easy recovery and purification. The purified His6-tagged fusion proteins appeared as a single band, and the fragment was adequately purified with apparent molecular weight of 60 kDa subjected to SDS-PAGE analysis (Figure 2A). The western blotting analyses (Figure 2B) showed that the host cells transformed by *ompA*-pET32a expressed an approximate molecular mass of 60 kDa protein detected by both anti-His_6 monoclonal antibodies and anti-OmpA polyclonal antibodies. This result was in agreement with the predicted size from the OmpA protein.

To test whether the recombinant OmpA protein could simultaneously induce a strong humoral response against bacteria, the serum antibodies were detected using ELISA. OmpA was found to induce high levels of antibodies recognizing predefined target protein (Figure 3). The first vaccination induced a significant increase in antibodies and after boosting for two weeks the antibody titer reached their

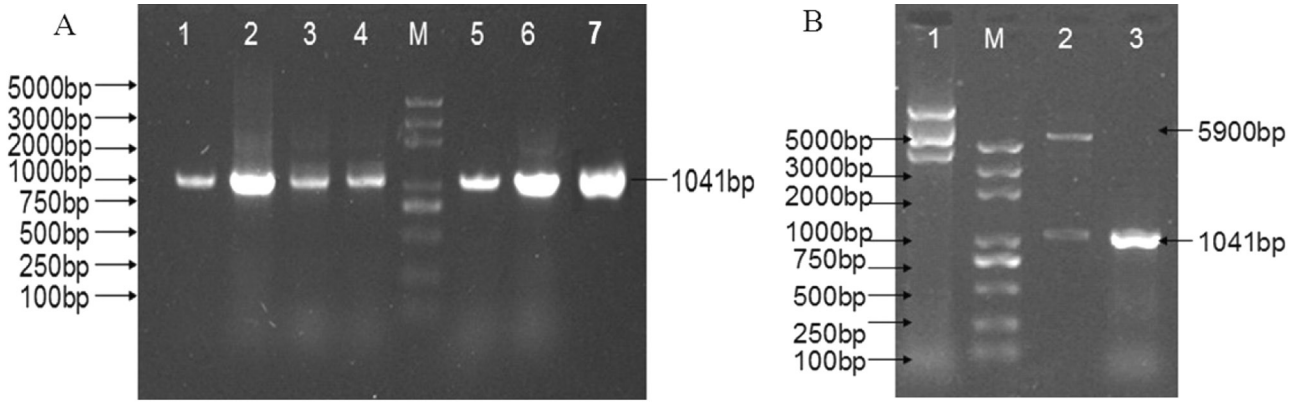

Figure 1. Amplification and cloning of *ompA* gene. (A) The *ompA* gene was amplified by PCR (1041 bp). Lane M: DNA Marker DL2K plus (Transgen, Beijing, China); lanes 1-7: *E. coli C83919*, *O157*, *J96*, *2002-1*, *K. pneumonia*; *S. flexneri* and *E. coli 308-2*. (B) Recombinant *ompA*-pET32a plasmid was identified with *Sal* I and *Hind* III (5900 bp and 1041 bp) digestion. Lane M: DNA Marker DL2K plus; lane 1: *ompA*-pET32; lane 2: *ompA*-pET32 digested with *Sac* I and *Hind* III; lane 3: *ompA*-pET PCR product (1041 bp).

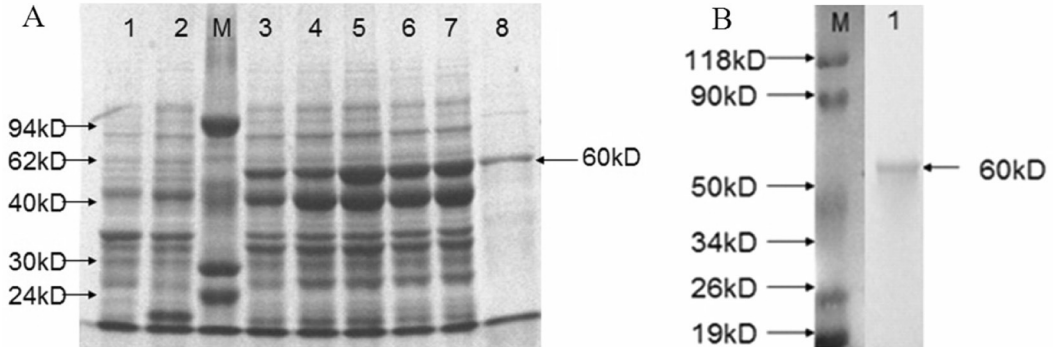

Figure 2. Expression and purification of the recombinant OmpA protein. (A) 12% SDS-PAGE showed expression and purification of recombinant OmpA protein. Lane Mprotein molecular weight marker (Takara, Dalian, China); lane 1: recombinant Rosetta cells without IPTG induction; lane 2: non-recombinant clone with 1mM IPTG induction for 6 h; lanes 3-7: recombinant clone with 1mM IPTG induction for 1-5 h, respectively; lane 8: purified recombinant OmpA protein (60 KDa). (B) Band visualized by western blotting using purified OmpA protein. Lane M: protein marker; lane 1: expression of OmpA.

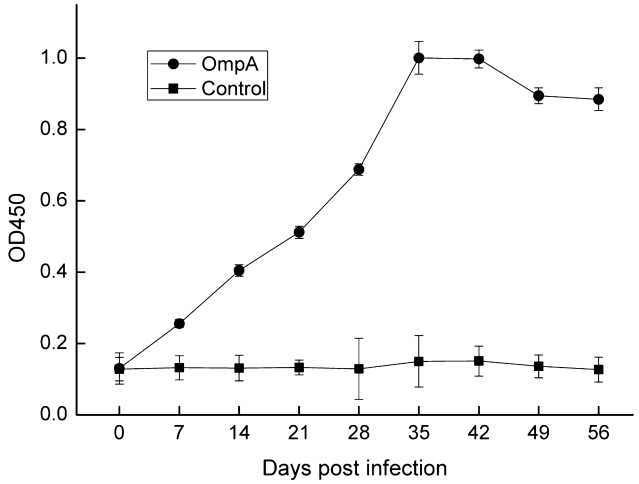

Figure 3. High level of predefined peptide-specific antibodies induced by OmpA.

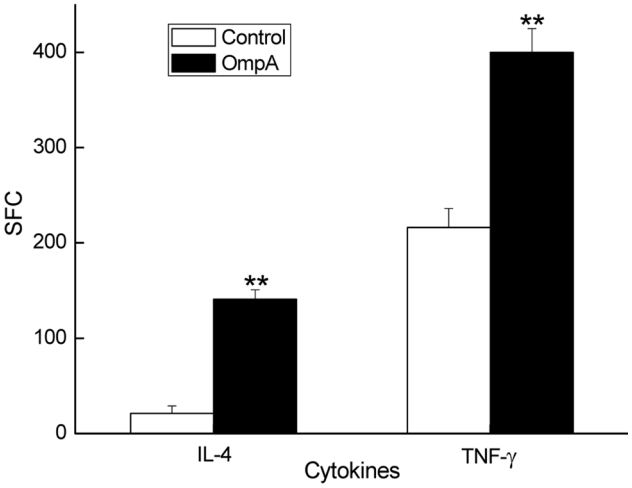

Figure 4. OmpA induced significantly higher levels of both IFN-γ and IL-4 cytokines in immunized animals (**P < 0.01).

highest titers of 1: 64,000. The levels then declined gradually until day 56. The antisera did not bind to the control peptide. Similarly, the normal mouse sera (pre-immune sera) did not bind with OmpA. These results indicated that the recombinant protein simultaneously induced high level of predefined OmpA antibodies.

The induction of IFN-γ and IL-4 cytokines was evaluated from splenocytes stimulated by antigens *in vitro* by ELISPOT analysis. Our results indicated that OmpA induced significantly higher levels of both IFN-γ (SFC=400) and IL-4 (SFC=141) cytokines in immunized animals, compared to IFN-γ (SFC=216) and IL-4 (SFC=21) cytokines in the PBS-treated controls ($p < 0.01$) (Figure 4). Furthermore, we found the induction pattern of both IFN-γ and IL-4 cytokines followed the same trend in all vaccinated groups without significant polarization.

Two weeks after boosting, control and OmpA-immunized mice were challenged using *E. coli C83919* (5×10^8 CFU), *O157* (1×10^8 CFU), *J96* (3×10^8 CFU), *2002-1* (5×10^8 CFU), *K. pneumonia* (1×10^5 CFU), and *S. flexneri* (5×10^8 CFU), respectively. The protective efficacy of the OmpA was evaluated in terms of survival number. As shown in Figure 5, none of challenged control animals survived in each separate experiment after a week post challenge. In comparison to control, more than 3 animals immunized with OmpA survived after challenge indicating the immunoprotective potential of OmpA. As expected, OmpA generated cross protection against *K. pneumonia* and *S. flexneri* challenge, which supported the evidence that *ompA* gene was amplified by PCR in *K. pneumonia* and *S. flexneri* genomic DNA.

DISCUSSION

Enterobacteriaceae infections have emerged as a significant veterinary clinical problem due to the increase caused by antibiotic-resistant strains (22, 23). There is therefore an urgent need to seek novel therapeutic strategies to combat *Enterobacteriaceae*. Hence, OmpA could contact with effectors of immune response such as antigen presenting cells and may be good candidates for vaccine development (7). This study explored the protective effect of outer membrane protein A (OmpA) against *Enterobacteriaceae* challenge in Balb/c mice and demonstrated that OmpA could serve as immunoprotective antigens against lethal *E. coli* infections. Besides, we found OmpA generated cross-protection against *K. pneumonia* and *S. flexneri* challenge, which implied that OmpA could be used as a potential vaccine for combating mixed infections of multiple Gram-negative *Enterobacteriaceae*.

PCR amplification showed *ompA* genes were present in the *E. coli 308-2*, *2002-1*, *C83919*, *O157*, *J96*, *K. pneumonia*, and *S. flexneri* isolates. More recently, the OmpA protein has been also characterized in *K. pneumonia* (24) and the other Gram-negative bacteria (25, 26, 27). These findings implied that *ompA* extensively existed in Gram-negative bacteria. The sequence of *ompA* gene from *E. coli 308-2* strains obtained in this study showed more than 98% of similarity among

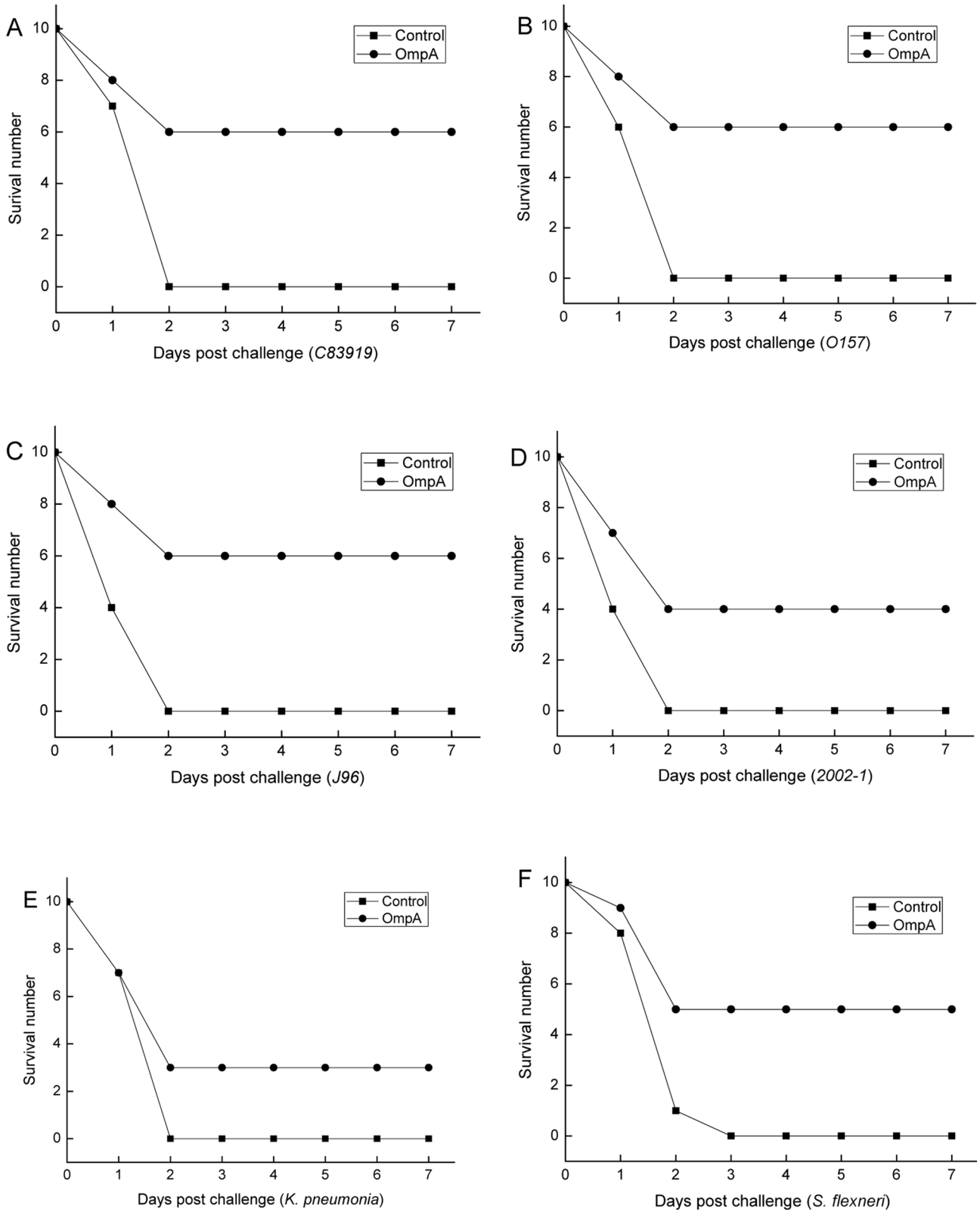

Figure 5. Survival number of animals challenged with lethal bacteria after OmpA immunization. Balb/c mice immunized with OmpA were challenged two weeks after the boost with six various bacteria, respectively. The animals were monitored for mortality till days 7 challenge. The data is representative of six experiments. (A) *E. coli C83919*; (B) O157; (C) *J96*; (D) *2002-1*; (E) *K. pneumonia*; (F) *S. flexneri*.

them (16). Moreover, recombinant OmpA protein containing His_6-tag was expressed with an average molecular weight of 60 kDa detected on 12% gel of SDS-PAGE (Figure 2). Our results of Western blotting further confirmed the presence of a major OmpA band at 60 kDa. OmpA were purified in soluble form to avoid the use of any denaturing agent and retain the high immunogenicity of the protein. The protective efficacy was then analyzed in animal experiments.

The immunogenicity of the Gram-negative-bacterial Omps has been assessed by several investigators. Jeannin *et al.* (7) who reported that OmpA appears in the form of a new type of pathogen-associated molecular pattern (PAMP) usable as a vector in anti-infectious and therapeutic anti-tumor vaccines to elicit cytotoxic T lymphocytes (CTLs). However, the data regarding OmpA expressed in Rosetta and its cross immunogenicity of the purified protein in mice has been very limited. In our study, Balb/c mice vaccinated with purified recombinant OmpA protein elicited a significant immunogenic response. High level of antibodies recognizing predefined target protein was induced by OmpA. At the same time, OmpA induced higher levels of both IFN-γ and IL-4 cytokines in immunized animals as compared to PBS-immunized controls. Furthermore, we have evaluated the protective potential of recombinant protein using a mice intraperitoneal challenge experiment.

More than 30% of the animals immunized with OmpA survived after lethal challenge, which demonstrated that an OmpA protein could impart a significant level of protection against *E. coli*, *K. pneumonia*, and *S. flexneri* as revealed by enhanced survival of immunized animals. In the same way, Kawai *et al.* (11) reported the immunogenic response of Omp and induction of protective immunity against *Edwardsiella tarda* infection in Japanese flounder. It is reported that Omp emerged as novel protective antigens which could induce a good protection against *Leptospira interrogans* challenge (12). These findings were similar to our findings in the cross protection against *K. pneumonia*, and *S. flexneri*, which suggested that the OmpA could be selected as a potential candidate for developing vaccine of Gram-negative bacteria.

In conclusion, our results demonstrated that OmpA is a promising immunogen which may be used for the development of a safe and effective vaccine against *Enterobacteriaceae* infection. Our study indicated the predominant immunological response of OmpA was antibody-mediated whereby the bacteria may be cleared by cytokine-mediated phagocytosis. Even so, it is possible that other mechanisms might also be responsible for the protection against *Enterobacteriaceae* infection and need to be further investigated.

ACKNOWLEDGEMENTS

This study was supported by National Natural Science Foundation of China (NSFC, Grant No. 31072120) and the Technology Research Foundation of Education Department of HeiLongJiang Province, China (12511352) and the Foundation of DaQing High-tech Industrial Development Zone (DQGX10ZS007). We are sincerely grateful to Hongqiong Zhao (Xinjiang Agricultural University & Obihiro University of Agriculture and Veterinary Medicine) for reading of the manuscript.

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