

## Two novel cross-protective antigens for bovine *Pasteurella multocida*

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**Abstract.** *Pasteurella multocida* is an important pathogen that leads to a range of diseases that have severe economic consequences on cattle production. In order to develop an effective cross-protective component vaccine, an immunoproteomics approach was used to analyze outer membrane proteins (OMPs) of the *P. multocida* serotype A, B and F strains. Candidate antigen molecules from the whole genome were screened via linear trap quadrupole mass spectrometry and bioinformatics analysis, and the reactogenicity of the candidate antigen molecules was analyzed via cloning, expression, and ELISA or protein immunoblotting, and the vaccine efficacy of the candidate molecules was determined in infective animal models and cross-protective antigens may be obtained. rPmCQ2\_2g0128, rPmCQ2\_1g0327 and rPmCQ2\_1g0020 proteins were selected. Their protective rates were 40/30/20% (rPmCQ2\_2g0128), 50/40/0% (rPmCQ2\_1g0327) and 0/40/30% (rPmCQ2\_1g0020) against ten-fold median lethal dose (10LD<sub>50</sub>) of the *P. multocida* serotypes A, B and F in a mouse model, respectively. The results suggested that the two proteins rPmCQ2\_2g0128 and rPmCQ2\_1g0327 may be used as vaccine candidates against bovine *P. multocida* serotypes A, B. To the best of our knowledge, the present study was the first to identify cross-protective antigens, extracted OMPs from bovine *P. multocida*.

### Introduction

*Pasteurella multocida* (*P. multocida*) is an important opportunistic pathogen that leads to a range of diseases in domestic and wild animals, including cattle (1), pigs (2), rabbits (3) and birds (4). *P. multocida* strains are classified into five serotypes: A, B, D, E and F, based on the capsular composition and 1-16 somatic serovars (5,6). The *P. multocida* serotypes A, B and F are the primary serotypes responsible for bovine pneumonia and hemorrhagic septicemia, and abscesses, otitis and septicemia in rabbits (7-9). In addition, the *P. multocida* serotype A:1 (Pm A:1) is associated with fatal pneumonia and septicemia in cattle and buffaloes (10), which are responsible for 30% of cattle deaths worldwide and \$1 billion in annual loss for beef cattle production in North America (11).

Vaccinations with inactivated bacteria (bacterins) or live attenuated bacteria is an effective and economic method for improving the condition of and protecting animals against *P. multocida* infections (11). However, *P. multocida* bacterins provide limited protection for heterologous serotype and the live/attenuated vaccines may revert to virulence, thus infecting animals (8). Therefore, the present study focused on *P. multocida* immunogens that elicit cross-protective immunity and may be used in vaccines against pasteurellosis (12). Outer membrane proteins (OMPs) provide protective immunity against *P. multocida* infection (13); therefore, OMPs may be primary protective antigens. Therefore, the *P. multocida* outer membrane proteome may aid in identifying potential vaccine candidates and diagnostic antigens. A high immunogenicity lipoprotein in *P. multocida*, PlpE, provided 80-100% protection in mice and induced cross-serotype protective immunity (14). The recombinant adhesive protein rCp39 provided high levels of protection from fowl cholera in chickens via challenge-exposure with the parental strain P-1059 or the heterologous strain X-73 (serotype A:1) compared with various other vaccines, e.g., PlpB and MOMPs (15). Iron-regulated OMPs (IROMPs) provided protection against *P. multocida* in rabbits (16) and calves (17,18). The recombinant OmpH-based vaccine provided cross-protection against avian *P. multocida* strains (15). These recombinant proteins may be used as subunit vaccines, which are safe and efficient for immuno-compromised animals; however, they provide limited protection against heterologous serotypes. Therefore, identifying cross-protective antigens

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may improve current vaccines. The present study was based on an immunoproteomic approach and identified two novel immunogenic OMPs with evident cross-protection against bovine *P. multocida* A, B and F strains. These two immunogenic OMPs may be promising candidates for a universal cross-protective vaccine that may have effective antigen variants. To the best of our knowledge, the present study was the first to identify cross-protective antigens extracted from OMPs in bovine *P. multocida*.

## Materials and methods

### *Bacterial strains and culture conditions, vectors, and primers.*

The bovine *P. multocida* serotype A PmCQ2 and PmCQ6 strains were isolated from lungs of feedlot calves suffering from fatal pneumonia at several cattle farms in Chongqing, China between May 2012 and December 2013. The bovine *P. multocida* serotype F PmF strain was isolated from lungs of feedlot calves suffering from disease of respiratory system at Gaojiazhen farms in Fengdu (Chongqing, China, longitude/latitude 107.70/29.89) in October 2013. The serotype B strain PmB is a well-characterized pathogenic strain and was purchased from the Chinese Veterinary Drug Supervision (cat. no. CVCC450; Beijing, China). The animal studies were performed with approval from the ethics committee of Southwest University (Beibei, China; permit no. 11-1025) and in compliance with the Laboratory Animal Care principles of the National Institutes of Health (Bethesda, MD, USA). The *P. multocida* strains were aerobically cultured in Martin's broth medium (Qingdao Hope Biol-Technology Co., Ltd., Qingdao, China) at 37°C for 12 h. *E. coli* DH5 $\alpha$  was aerobically cultured in Luria Bertani broth (LB; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 37°C for 20 h. The *E. coli* strain genomic DNA was used as a negative control.

*Mice and housing conditions.* A total of 120 Female Kunming (KM) mice (8 weeks-old, body weight 18–22 g) were obtained from the Chongqing Academy of Chinese Materia Medica (Chongqing, China). The animals were housed in pathogen-free conditions (temperature, 20–30°C; relative humidity, 45–60%; 12-h light/dark cycle) and were given free access to food (standard rodent diet) and drinking water.

*OMP preparation.* OMPs were prepared in accordance with previously described protocols (19–21). The bacteria cell cultures were harvested by centrifugation at 10,000  $\times$  g for 15 min at 4°C. The pellets were washed 3 times with cold, sterile PBS (pH 7.4) and resuspended in PBS (pH 7.4) containing 8% Triton X-114, 0.5% 3-(3-cholamidopropyl)-dimethylammonio-1-propanesulfonate and 1 mmol/l phenylmethylsulfonyl fluoride, and maintained at 4°C for 3 h. The cell debris was removed via centrifugation at 10,000  $\times$  g and 4°C for 10 min, and the aqueous phase was collected and 10  $\mu$ g OMPs were detected by using 12% SDS-PAGE gel. The gelatinous decontamination phase was added into 1:9 volume absolute ethanol, and the pellets were collected by centrifugation at 10,000  $\times$  g and 4°C for 30 min, washed twice with PBS, and dissolved into PBS. Concentration of OMPs was determined using the Bradford method (Bradford Protein Assay kit; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

*Western blot analysis and linear trap quadrupole (LTQ)/mass spectrometry (MS).* The 10  $\mu$ g OMPs were separated by using 12% SDS-PAGE gel and blotted electrophoretically onto a polyvinylidene difluoride membrane. The membrane was incubated with infected PmCQ2 bovine serum (PmCQ2-IS; 1:16) and horseradish peroxidase-conjugated rabbit anti-bovine IgG antibodies (cat. no. SAB3700018; 1:20,000; Sigma-Aldrich; Merck KGaA). An Enhanced Chemiluminescent reagent (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was applied for a chromogenic reaction. Proteins were visualized using a LTQ Mass Spectrometer (Shanghai Applied Protein Technology Co. Ltd., Shanghai, China). Proteins in *P. multocida* species were detected using BioWorks Browser software version 3.3 (bioworks.software.informer.com).

*Protein expression and purification.* The target genes were amplified from the genomic DNA of strain PmCQ2. DNAPol was from Takara Biotechnology Co., Ltd. (Chongqing, China) and the primers for the selected genes are presented in Table I. The products of polymerase chain reaction (94°C for 3 min, 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, 35 cycles at 72°C for 10 min, stored at 4°C) were cloned into pET-28a plasmid (Takara Biotechnology Co., Ltd.) in *E. coli* DH5 $\alpha$  (Takara Biotechnology Co., Ltd.), expressed in *E. coli* BL21 (DE3; Takara Biotechnology Co., Ltd.), cultured at 37°C in LB broth or on agar supplemented with 100  $\mu$ g/ml kanamycin (Sigma-Aldrich; Merck KGaA) and induced with 0.5 mM isopropyl  $\beta$ -D-1-thiogalactopyranoside (Sigma-Aldrich; Merck KGaA) at 30°C or 37°C for 4 h. The recombinant proteins were purified using affinity chromatography and Ni-NTA Superflow cartridges (Qiagen GmbH, Hilden, Germany).

*Anti-serum preparation.* A total of 4 *P. multocida*-antibody-free calves were purchased from a farm in Chongqing. One calf was immunized intramuscularly in the neck with 4.8 $\times$ 10<sup>10</sup> colony forming unit (CFU)-inactivated PmCQ2, which was fully emulsified with an equal volume of Freund's incomplete adjuvant (Sigma-Aldrich; Merck KGaA) following 3 administrations with 10-day intervals between each. The blood was collected for anti-serum (PmCQ2-VS) separation. A second calf was intramuscularly injected in the neck with 4.8 $\times$ 10<sup>10</sup> CFU live PmCQ2. Following 3 injections with the same dose at 10-day intervals for each dose, the antisera (PmCQ2-IS) obtained from the calf were separated post-infection at 30 days. The remaining two calves were intranasally infected with 4.8 $\times$ 10<sup>10</sup> CFU live PmB and PmF 3 times. The calves were injected 3 times with the same dose, at 10 day intervals for each dose. Subsequently, the antisera (PmB-IS and PmF-IS) were separated post-infection at 30 days.

*Indirect ELISA.* Recombinant protein was dissolved in coating buffer (0.5 M carbonate buffer; pH 9.6) and added to the well (10  $\mu$ g protein/well) of 96-well microplates (Nunc-Immuno™ MicroWell; Merck KGaA) and then incubated at 4°C overnight. Plates were washed three times with PBS containing 0.05% Tween 20 (PBST), blocked for prevention of non-specific binding with 200  $\mu$ l 2% skimmed milk (Sigma-Aldrich; Merck KGaA) at 37°C for 1 h. A 1:100 dilution of bovine serum in blocking buffer was added to each well and incubated at 37°C for 1 h. Following washing three times each

Table I. Primer sequences for the selected genes.

Gene	Forward (5'-3')	Reverse (5'-3')	Length
<i>PmCQ2_3g0306</i>	CCGGAATTCGCACCACAACCTAAC	CCGCTCGAGTTATTTGTTACCTTTAACAGCG	962
<i>PmCQ2_2g0128</i>	CCGGAATTCGAAAGTAAACA GCAAGC	CCGCTCGAGTTATTTGCTAAGAGATTTTG TAATGC	951
<i>PmCQ2_1g0327</i>	CCGGAATTCGGTGGGGGTAATAAC	CCGCTCGAGTTATTGTTTTTCTCCATACCTA AAACACCCC	948
<i>PmCQ2_3g0121</i>	CCGGAATTCGGTGCACCAAGTAGC	CCGCTCGAGTTAATCCGCTAACTTAGTGC	1,500
<i>PmCQ2_2g0046</i>	CCGGAATTCGTTGAGCTCGAAAAC	CCGCTCGAGTTAAATGCGTGATTTCG	975
<i>PmCQ2_1g0020</i>	CCGGAATTCGGCACCGAATTAGCC	CCGCTCGAGGAAGTTCTTACCAGGAT	1,518

Underlined bases represent the double enzyme loci of *EcoRI/XhoI*.

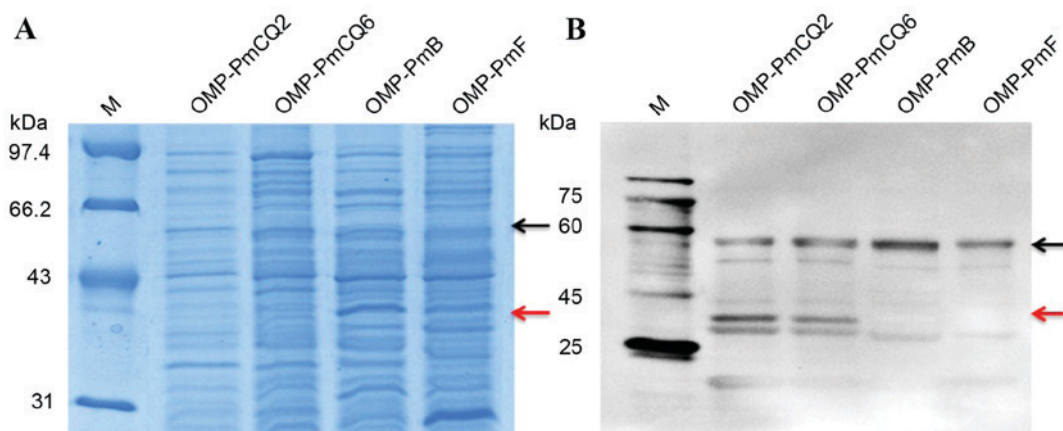


Figure 1. OMPs of *P. multocida* serotypes A, B and F. (A) SDS-PAGE of the extracted OMPs fragments. (B) Representative western blot images for OMP expression. Black arrows indicate the common band in *P. multocida* serotypes A, B and F. Red arrows indicated a unique band in *P. multocida* serotype A. OMP, outer membrane protein; M, marker; *P. multocida*, bovine *Pasteurella multocida*.

for 20 min with PBST, a 1:20,000 dilution of horseradish peroxidase-conjugated rabbit anti-bovine IgG antibodies (SAB3700018; Sigma-Aldrich; Merck KGaA) in blocking buffer was added to each well and incubated continuously at 37°C for 1 h, then washed three times each for 20 min with PBST. Then, 100  $\mu$ l substrate solution [30 mg o-phenylenediaminedihydrochloride (Takara Biotechnology Co., Ltd.) in 100 ml substrate buffer (0.2 M  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ ; 0.1 M  $\text{C}_6\text{H}_8\text{O}_7$ ; pH 4.8)] was added to each well and incubated without light for 15–30 min at room temperature. The reaction was stopped by adding 100  $\mu$ l 2N sulfuric acid ( $\text{H}_2\text{SO}_4$ ; Sigma-Aldrich; Merck KGaA) and absorbance values were recorded at a wave length 450 nm by ELISA reader (Spectrophotometer 1510, Thermo Fisher Scientific, Inc.).

**Mice immunization and challenge treatments.** A total of 120 female KM mice were equally divided into 12 groups (n=10/group). Each mouse was subcutaneously inoculated with the 100  $\mu$ g/dose recombinant proteins. The control group mice were inoculated with 100  $\mu$ l PBS. The initial two inoculations occurred once in two consecutive weeks and the third and final inoculation occurred in week 4. The mice were intramuscularly injected with 10  $\text{LD}_{50}$  PmCQ2 ( $\text{LD}_{50}=6.48 \times 10^6$  CFU),

PmB ( $\text{LD}_{50}=3.87 \times 10^6$  CFU) and PmF ( $\text{LD}_{50}=1 \times 10^7$  CFU) strains 2 weeks after the third and final inoculation.

**Statistical analysis.** Data were analyzed by Microsoft Office Excel 2010 and SPSS version 20 (IBM SPSS, Armonk, NY, USA). Sequence homology analysis was employed using ClustalW2 (<http://www.ebi.ac.uk/tools/clustalw2>). Survival rates of animals were evaluated using Kaplan-Meier analysis and Life test of SAS 9.2 (SAS Institute Inc.)  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**OMP extraction and western blot analysis.** The OMPs were extracted using Triton X-114 from *P. multocida* serotype A, B and F strains. The OMPs were identified by SDS-PAGE (Fig. 1A) western blot analysis (Fig. 1B) of PmCQ2-IS serum. A ~57 kDa common OMP band was observed in PmCQ2, PmCQ6, PmB and PmF (indicated by black arrows in Fig. 1), and a clear 34 kDa OMP band in PmCQ2 and PmCQ6 (indicated by red arrows in Fig. 1). To identify the candidate cross-protective antigens for *P. multocida* serotype A, the two gel bands were cut out and used for LTQ/MS analyses.



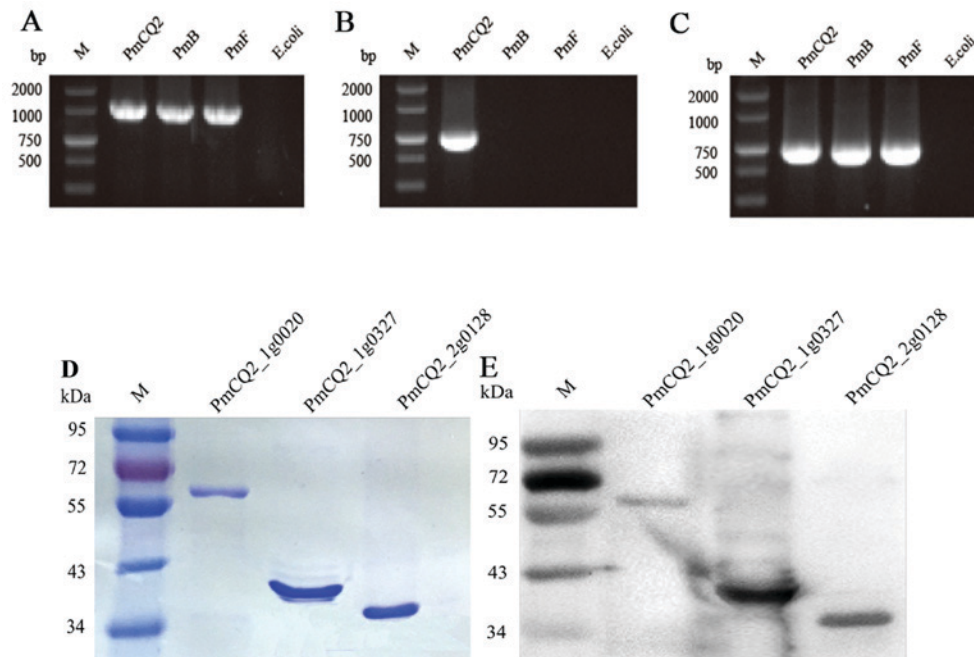


Figure 2. PCR amplification and purification of recombinant proteins. A 1.0% agarose gel showing PCR-amplified gene products of (A) *PmCQ2\_2g0128*, (B) *PmCQ2\_1g0327* and (C) *PmCQ2\_1g0020* gene products of PmCQ2. (D) Purified 3 recombinant protein fragments. (E) Representative western blot image of polyclonal sera (PmCQ2-IS). PCR, polymerase chain reaction; M, DL2000 DNA marker.

**LTQ/MS and bioinformatics analysis.** Based on the LTQ/MS analysis, the protein sequences were compared with the Uniprot database ([www.uniprot.org](http://www.uniprot.org)) and our bovine *P. multocida* genome database. A total of 6 protein sequences were matched and are presented in Table II. These protein sequences were predicted as secretory proteins using TMHMM Server.2.0 (22), SignalPv3.0 (23), PSORTb (24) and GPI-SOM (25). A total of 6 protein sequences contained a signal peptide, and 3 secretory proteins were predicted from the bioinformatics analysis (Table I). Using the aforementioned combined analyses, 6 protein sequences were cloned and purified.

**Recombinant protein selection.** The sera for PmCQ2-VS, PmCQ2-IS, PmB-IS and PmF-IS were used to test the reactivities of 6 recombinant proteins using an indirect ELISA (Table III). rPmCQ2\_3g0306, rPmCQ2\_2g0128, rPmCQ2\_3g0121 and rPmCQ2\_2g0046 had the high reactivity to PmCQ2-IS; however, only rPmCQ2\_1g0327 and rPmCQ2\_1g0020 were able to react with the 4 sera. Therefore, the present study used r-PmCQ2\_2g0128, r-PmCQ2\_1g0327 and r-PmCQ2\_1g0020 to verify potential cross-protective antigens and diagnostic antigens for *P. multocida* serotype A.

**Characterization and protective efficacy of recombinant proteins.** The present study extracted genomic DNA from the PmCQ2, PmB, PmF and *E. coli* strains to amplify recombinant proteins (Fig. 2). *PmCQ2\_1g0020* and *PmCQ2\_2g0128* DNA were amplified in PmCQ2, PmB and PmF (Fig. 2A and C, respectively). However, *PmCQ2\_1g0327* DNA was not amplified in PmB and PmF (Fig. 2B). The purity of recombinant proteins was determined using SDS-PAGE, which revealed single bands with the molecular masses 34, 35 and 57 kDa (Fig. 2D). The identity and integrity of the recombinant proteins

(rPmCQ2\_1g0020, rPmCQ2\_1g0327 and rPmCQ2\_2g0128) were determined using western blotting (Fig. 2E).

The homology analysis was performed with Clustal X software version 2.0 (<http://www.ebi.ac.uk/tools/clustalw2>) on *PmCQ2\_1g0327* and different *P. multocida* strains that contained PmCQ2, PmCQ6, PmB, PmF, 5 additional bovine *P. multocida* (isolated from the lungs of diseased cattle in our laboratory), 1 poultry *P. multocida* (isolated from the lungs of diseased anseriformes in our laboratory) and 27 additional *P. multocida* genome sequences from the National Center for Biotechnology Information ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). Sequence analysis revealed that *PmCQ2\_1g0327* exhibited a 100% sequence match to *Pmu\_17770* and *PmCQ6\_c4301g0004* in *P. multocida* serotype A Pm36950 and PmCQ6 strains (Fig. 3).

In order to evaluate the protective efficacy of these proteins, mice were immunized with 100  $\mu$ g/dose purified r-PmCQ2\_2g0128, r-PmCQ2\_1g0327 and r-PmCQ2\_1g0020. Therefore, it was determined that up to 7 days after a challenge with PmCQ2, r-PmCQ2\_1g0327 and r-PmCQ2\_2g0128 proteins provided improved survival rates (50 and 40%, respectively) compared with PBS alone (control group) and r-PmCQ2\_1g0020 (0%). No statistically significant difference was identified between the protection percentages (30-40%) of these three proteins against PmB. However, the protection percentages against PmF were low or zero (Fig. 4).

## Discussion

Pasteurella is a major respiratory infection in farm and wild animals that has various symptoms, such as swine plague, bovine pneumonia, hemorrhagic septicemia in cattle and abscesses, otitis, and septicemia in rabbit, cholera fowl and

Table II. Linear trap quadrupole mass spectrometry and bioinformatics analysis.

Protein	Common band	Unique band	Signal peptides	Transmembrane domains	Secretory protein	Description
PmCQ2_3g0306	2	222	Y	N	Y	Outer membrane protein A
PmCQ2_2g0128	0	111	Y	N	N	Outer membrane protein assembly factor BamC
PmCQ2_1g0327	0	31	Y	N	N	Putative transmembrane protein
PmCQ2_3g0121	10	0	Y	N	Y	Heme-binding protein A
PmCQ2_2g0046	0	7	Y	N	N	Glycosyl transferase, family 2 protein
PmCQ2_1g0020	7	0	Y	N	Y	Peptide ABC transporter substrate-binding protein

Common band is ~58 kDa band in three serotype A, B, F strains, the unique band is ~35 kDa band which was only in the *P. multocida* serotype. The value in table represents the extent of the match peptides. Y, yes; N, no.

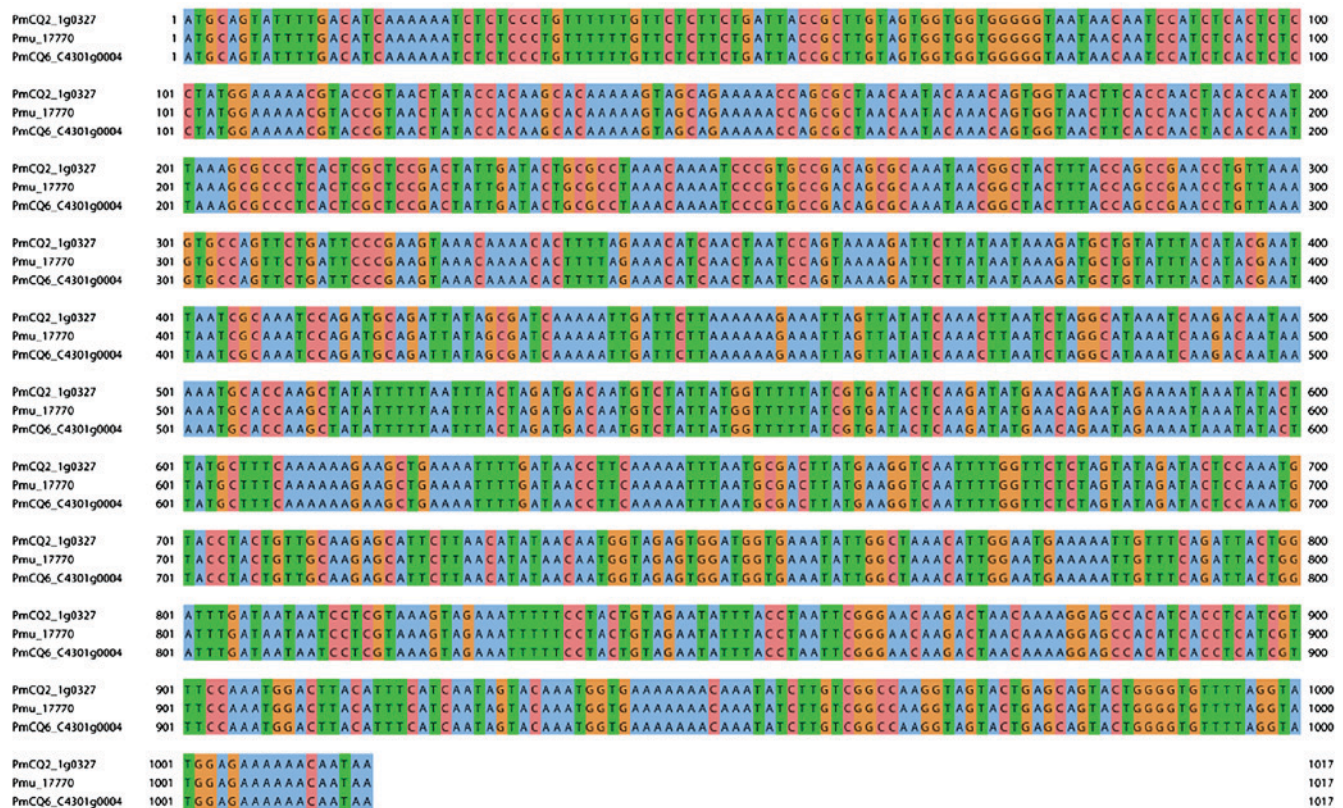


Figure 3. Sequence homology analysis for PmCQ2 (*PmCQ2\_1g0327*), 36950 (*Pmu\_17770*) and PmCQ6 (*PmCQ6\_C4301g0004*). The bovine *Pasteurella multocida* serotype A strains PmCQ2 and PmCQ6 were isolated from the lungs of diseased cattle. The bovine respiratory disease isolate 36950 (GenBank: NC\_016808.1) was downloaded from the NCBI.

swine atrophic rhinitis (1-4). *P. multocida* is the opportunistic pathogen responsible for this contagious disease. At present, commercially available vaccines are live attenuated vaccines and bacterin vaccines. The bacterins of *P. multocida* provided limited protection to heterologous serotypes and the live or attenuated vaccines may reverse the virulence of the animal disease. Based on the inevitable disadvantages, it is challenging to develop novel vaccine formulations which are effective, safe, have low production costs, and practicality

for herd health (26). Bacterial OMPs are highly immunogenic and expose epitopes in the *P. multocida* envelope, which are important for conferring protective immunity in a range of infection models. Previous studies determined that the avian *P. multocida* PlpB protein was a cross-protective antigen (27), and this protein was located in the *P. multocida* outer membrane (28). The present study extracted OMPs from the different bovine *P. multocida* strains. Two protein bands were identified (30-40 kDa special bands



Table III. Positive to negative value (P/N) ratios of the 6 recombinant proteins in the indirect ELISA.

P/N	PmCQ2-IS	PmCQ2-VS	PmB-IS	PmF-IS
rPmCQ2_3g0306	5.53	1.45	2.50	1.98
rPmCQ2_2g0128	6.97	1.87	2.11	1.56
rPmCQ2_1g0327	8.22	7.68	8.71	10.06
rPmCQ2_3g0121	3.36	1.83	0.89	1.12
rPmCQ2_2g0046	5.35	1.47	1.35	1.29
rPmCQ2_1g0020	6.92	6.05	6.28	8.52

and 50-60 kDa common bands) by western blot analysis of PmCQ2-infected serum. Based on the LTQ/MS analyses, 6 proteins were cloned, expressed, purified and used for an indirect ELISA. Based on the P/N values, it is possible that r-PmCQ2\_3g0306, r-PmCQ2\_2g0128, r-PmCQ2\_3g0121 and r-PmCQ2\_2g0046 represent potential diagnostic antigens for the *P. multocida* serotype A, based on high P/N values with PmCQ2-IS. The r-PmCQ2\_1g0327 and r-PmCQ2\_1g0020 antigens may be potential cross-protective antigens due to the high P/N values for the different serotypes A, B and F. Subsequently, 3 proteins were selected for evaluation of their protective efficacy in mice. The findings of the present study revealed that the r-PmCQ2\_1g0327 and r-PmCQ2\_2g0128 exhibited 40-50% survival rates and may induce cross-immunity in mice against the *P. multocida* serotype A and B strains. The homology analysis revealed that PmCQ2\_1g0327 exhibited 100% sequence identity with three *P. multocida* serotype A strains; therefore, PmCQ2\_1g0327 may be used as a diagnostic antigen for *P. multocida* serotype A strains. However, the function of this protein requires further investigation.

B-cell epitopes have an important role in vaccine development and disease diagnosis (29,30). Epitope identification provides valuable information that is essential for understanding antigen components (31). The present study did not identify PmCQ2\_1g0327 in the PmB and PmF genomes; however, r-PmCQ2\_1g0327 protected against a PmB infection challenge. Therefore, the present study identified the B-cell epitopes for associated genes in PmCQ2 and PmB using the Protean software and the DNASTar package. The findings revealed specific antigenic epitopes, which were similar to r-PmCQ2\_1g0327 in PmCQ2 and PmB.

In conclusion, r-PmCQ2\_1g0327 and r-PmCQ2\_1g0020 may serve as cross-protective antigens or effective vaccine components against infection with *P. multocida* serotypes A and B. However, the two recombinant proteins require additional investigation to verify protective immunity in cattle and confirm potential cross-protective antigens to develop *P. multocida* vaccines. In addition, the protective immunity of the residual proteins (r-PmCQ2\_3g0306, r-PmCQ2\_3g0121 and r-PmCQ2\_2g0046) require investigation, as they exhibit higher OD values with the live PmCQ2 infected serum and not the inactivated PmCQ2 immune serum. Therefore, these proteins may serve as diagnostic antigens for *P. multocida* serotype A strains. Additionally, the ELISA assay platform

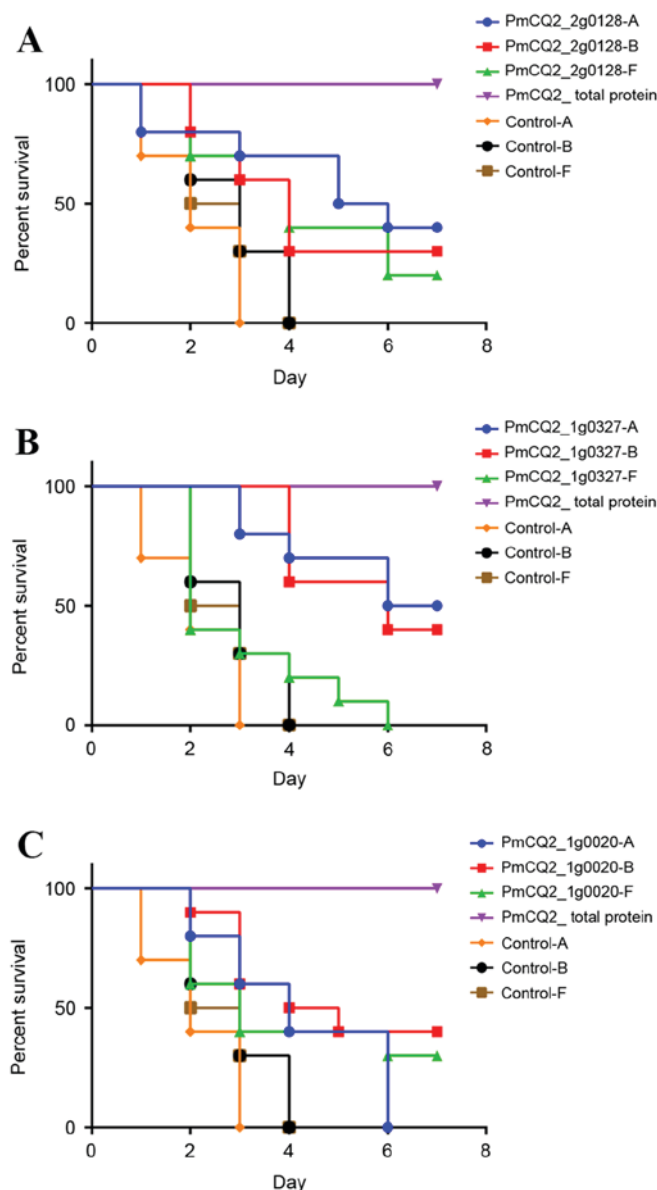


Figure 4. Protective efficacy of the recombinant proteins in mice model. (A) r-PmCQ2\_2g0128, (B) r-PmCQ2\_1g0327 and (C) r-PmCQ2\_1g0020 were used to immunize the mice at a dosage of 100  $\mu$ g/mice. The percentage survival curve revealed the survival/mortality pattern for immunized and control mice (10 mice/group) following a challenge with 10LD<sub>50</sub> PmCQ2, PmB and PmF. PmCQ2\_total protein was used as a positive control.

with specific sera (three serotypes) was a useful tool for the selection of protective and diagnostic antigens.

A previous study suggested that identifying OMPs may be important in the development of novel vaccine candidates and diagnostic antigens (32). Therefore, future studies should focus on the development of novel vaccines for *P. multocida* strains, and the generation of fusion proteins that may provide improved protection against different *P. multocida* strains.

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