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Original article

Evaluations of Alkyl hydroperoxide reductase B cell antigen epitope as a potential epitope vaccine against *Campylobacter jejuni*

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ABSTRACT

Objective: The present study aimed to screen and find alkyl hydroperoxide reductase (AhpC) B cell dominant epitope of *Campylobacter jejuni* (*C. jejuni*).
Materials and methods: Bio-informatic algorithms were used to predict B cell epitopes of AhpC. The AhpC protein and chemically synthesized antigenic epitopes of *C. jejuni* were considered as antigens, and the AhpC antibody was used as the primary antibody, ELISA and dot blot were used to analyze and screen the dominant epitope. The specific IgG of mice serum and IL-4 in splenocyte culture supernatant were detected by ELISA. The protective efficacy was evaluated by animal disease index and tissue histopathological staining of the jejunum.
Results: Seven epitopes of AhpC were predicted, one epitope ($AhpC_{4–16}$) was found to recognize the antibodies of AhpC and had strong antigenicity by ELISA and dot blot analysis. In epitope $AhpC_{4–16}$ immunized mice, specific IgG of serum and IL-4 in splenocyte culture supernatant were significantly higher. The illness index decreased significantly, the protective rate was 66.67%. Histopathology displayed that the jejunum morphology was better than the control group.
Conclusions: These findings suggested that epitope $AhpC_{4–16}$ showed effective protective role against *C. jejuni* and is a candidate epitope of vaccine against this pathogen.

1. Introduction

Since the isolation of *Campylobacter jejuni* (*C. jejuni*) by Dekeyser in 1968, intestinal *Campylobacter* infections are reported to be highly prevalent in developed countries. Most cases with *Campylobacter* infections are caused by *C. jejuni*, possessing 90% of this disease (Mansfield et al., 2007). *C. jejuni*, a Gram-negative rod, is a zoonotic pathogen, especially in human enteric infection. It usually causes enterocolitis and remains a major public health concern, as over 450 million people are infected with this pathogen every year worldwide (Schielke et al., 2014; Kumar et al., 2017).

Although many efforts have been put forwarded to understand the virulence determinants of *C. jejuni* in nature, limited progress has been achieved. By obtaining the strategies from the treatments of other similar diseases, vaccine remains to be a cost effective and safe way to combat diseases bacterial infections (Moriel et al., 2008). Alkyl hydroperoxide reductase (AhpC) belongs to the family of *C. jejuni* antioxidant enzymes, with a molecular mass of 22 kDa. It is ubiquitous in *C. jejuni* and is highly conserved, stable and specific protein (Seaver and Imlay, 2001; Oh and Jeon, 2014). Thus, AhpC is considered as an ideal target antigen to explore candidate antigens and prepare monoclonal antibodies of *C. jejuni* vaccine (Perkins et al., 2014). Hence, in this study, we designed several truncated peptides of AhpC by using bioinformatic algorithms, screened them and established the dominant epitope. We confirmed the immunoprotective effect by immunizing animals and finally providing a basis for the development of *C. jejuni* multiple antigenic peptide (MAP) vaccine.

2. Materials and methods

2.1. Bacteria and animals

C. jejuni was obtained from China center of industrial culture collection (CICC), with the conservation number CICC 22936

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(=ATCC 33291). Specific-pathogen free grade male rabbits and female BALB/C mice were purchased from Shanghai Jiesijie Laboratory Animal Co., Ltd. All animal-related operations were performed according to the Institutional Animal Care and Use Committee, and all efforts were made to minimize pain and suffering. The study was approved by the Medical Ethics Committee of Jinhua Polytechnic (Jinhua, PRC).

2.2. Prediction of B cell epitopes

Gene sequence of AphC (NC_002163.1) in *C. jejuni* strain was obtained from NCBI. Using SignalP 4.1, AhpC was predicted to have no signal peptide. Through TMHMM 2.0 analysis, no transmembrane sequence was shown in AhpC sequence. IEDB Analysis Resource was used to predict AhpC B-cell epitopes. The accession number of AhpC is AAD02329.1.

2.3. Recombinant protein and peptides

Complementary oligonucleotides corresponding to the amino acid sequences of AhpC were cloned into *BamHI-XhoI* digested pGEX-4T-1 plasmid. The glutathione-S-transferase (GST) tag was added to the C terminal of AhpC protein as a fusion protein, facilitating the purification of the protein. Recombinant plasmids were then transformed into the *E. coli* strain Rossetta (DE3) separately and the expression of recombinant AphC protein was induced by 0.5 mmol/L isopropyl β-D-galactopyranoside (IPTG) for 4 h. Then, the bacterial lysate was collected and the expression of AphC protein was detected by SDS-PAGE electrophoresis after purification using GST Sefinose™ Resin (Sangon Biotech Co., Ltd. Shanghai, PRC). All bands in the SDS-PAGE gels indicated purified fractions from cell lysates. Truncated peptide synthesis was performed by solid-phase peptide synthesis. As confirmed by HPLC purification and mass spectrometry, the purity of the peptides was more than 95%, and this work was completed by China Peptides Biotechnology Co., Ltd.

2.4. Preparation of anti-AhpC serum

To prepare anti-AhpC antibodies, three male rabbits were immunized with purified full length AhpC (200 μg) emulsified in the complete Freund's Adjuvant (1:1, Sigma-Aldrich, Merck KGaA, Darmstadt, FRG). Subsequent boosters were administered every two weeks (four times in total, 100 μg AhpC protein) with incomplete Freund's Adjuvant (Sigma-Aldrich) instead of complete Freund's Adjuvant. Rabbits were anesthetized using pentobarbital (30 mg/kg, intravenous), and then the blood was collected from the heart 10 days post the final immunization. Serum was separated from the collected blood and IgG in the serum was enriched by saturated ammonium sulfate precipitation and DEAE-52 column chromatography (Sangon Biotech). These were then named as purified antibodies 1, 2, and 3 respectively.

2.5. Dot blot

To reveal cross-reactivity between different truncated peptides and purified antibodies, 2 μg of each peptide was dropped separately on to a nitrocellulose membrane. The nitrocellulose membrane was naturally dried after a few minutes, and then placed into a blocking solution for 1 h at room temperature. After discarding the block solution, purified antibodies were added and kept on a shaker for 1 h. The membrane is then washed with 1 × TBST 3 times for 5 min, transferred into reaction slot with a secondary antibody (HRP-linked goat anti-rabbit IgG, 1:4000, ZSGB-BIO, Beijing, PRC), and incubated on a shaker for 1 h. After further 3 wash in TBST, the membrane was soaked with SuperSignal™ West Dura Extended Duration substrate (Thermo Fisher Scientific, Waltham, MA, USA), and exposed to X-ray film subsequently.

2.6. ELISA

Diluted truncated peptides (1 μg/mL) were coated in a 96-well plate and incubated at 4 °C overnight. Then the liquid was removed and the wells were washed by 1 × TBST. To block the nonspecific sites, 1% BSA (1 × TBST) was added into each well and incubated at 37 °C for 1 h. Each well was washed three times with 1 × TBST, followed by addition of purified antibodies with various dilutions (1:200, 1:1000, 1:5000, 1:10,000, 1:20,000, 1:60,000, 1:240,000), and incubation at 37 °C for 1 h. After washing again twice as described above, fresh HPR conjugated antibody (goat anti-rabbit IgG, 1:10,000, ZSGB-BIO, Beijing, PRC) was added into each well and incubated at 37 °C for 45 min. Then, the wells were washed once more and incubated with the 3,3′,5,5′-tetramethylbenzidine substrate solution for 5 min. Stop Solution was added to terminate the reaction, and immediately measured the absorbance using an microplate reader (680 Bio-Rad, Hercules CA, USA) at 450 nm.

2.7. Animal immunization

Forty-five BALB/C mice were randomly divided into three groups, including the control group, the $AhpC_{4-16}$ immune group and the AhpC immune group. Mice were given subcutaneous injection inoculation every week and received four times in total. Antigens were given in half volume of complete Freund's Adjuvant (Sigma-Aldrich) for the first time, which was instead by incomplete Freund's Adjuvant (Sigma-Aldrich) in the remaining times. For each injection, 0.25 μg antigen was used.

2.8. Measurement of immune responses

Two weeks after the final immunization and after completing the animal immunization process, animals were anesthetized with pentobarbital (50 mg/kg, intraperitoneal), and blood was collected from the hearts (5 mice per group). Serum was separated from each blood sample and ELISA measurement was performed to test the enrichment of IgG in the serum. To test the immune responses after immunization, spleens were obtained from the immunized mice. Lymphocytes separated from the spleens were cultured in 96-well plates at a concentration of 5×10^5 per well, and then co-cultured with ConA (5 μg/mL; 50 μL each well) and lysates from *C. jejuni* (50 μL per well) at 37 °C for 24 h. The suspensions from each cultured well were then collected to test the concentration of IFN-γ, and IL-4 by ELISA Kits (CUSABIO BIOTECH, Wuhan, PRC) according to the manufacturer's instructions.

2.9. Challenge with C. jejuni

C. jejuni was recovered by inoculation to fermentor, cultured under anaerobic conditions at 42 °C for 48–72 h, and then expanded under same conditions. Following the animal immunization process, mice (10 per group) were infected with *C. jejuni* (5×10^9 CFU) by irrigation after four weeks of the final immunization (day 1). After that infected again on days 3 and 7, and sterile sodium bicarbonate (10%, 100 μL) was used to neutralize stomach acid before *C. jejuni* irrigation. Observation was performed every day after the first infection until the eighth week post-immunization. The vaccine protection rate was calculated using the formula, vaccine protection rate = (control group illness index − immune illness index)/control group disease index × 100%.

2.10. Histopathological staining

After 8 weeks of last immunization, mice were anesthetized with pentobarbital and sacrificed by cervical dislocation. The jejunal tissues were taken and fixed in 4% neutral buffered paraformaldehyde and embedded in paraffin. Paraffin sections (3 mm) were cut and then stained with hematoxylin and eosin. All sections were evaluated blindly under light microscope for three histologic features regarding the mucosal damage and the extent of inflammation: mild, moderate or severe, as previously described (Jain et al., 2008).

2.11. Statistical analyses

The data were processed by SPSS 17.0 statistical software (IMB Corp., Armonk, NY, USA). Measurement data are expressed as means ± standard deviation. Data were analyzed by a one- or two-way ANOVA. Post hoc analyses were carried out by the Student-Newman-Keuls (SNK) Multiple Comparison Test. For univariate analysis, the classification variables were analyzed using χ^2 test and Mann–Whitney U test, whereas the continuous variables were analyzed using the paired t test. The differences between the groups were considered to be statistically significant when $P < 0.05$.

3. Results

3.1. Predicted B cell linear epitopes

Full gene sequence of AphC (NC_002163.1) in *C. jejuni* strain (NCTC11168) was obtained from NCBI. We initially used SignalP 4.1 to predict the signal peptides in AhpC and the results showed no signal peptide prediction. Then through TMHMM 2.0 analysis, we were unable to obtain transmembrane sequence in AhpC sequence. Then IEDB was used to predict AhpC B cell epitopes. Seven B cell epitopes of AhpC protein were predicted. The names and amino acid sequences of these peptides were displayed in Table 1. Cysteine residue was added to the end of each sequence to cross-link with KLH. All the peptide purities were above 95% and qualities were examined by HPLC-MS method.

Table 1
Prediction of *C. jejuni* AhpC linear B cell epitope sequence.

Name	Sequence	Length
$AhpC_{34-57}$	KGAVVFFYPKDFTFVCPSEIIAFD	24
$AhpC_{94-108}$	IGQVKFPLVADLTKQ	15
$AhpC_{112-132}$	NFDVLYAEAVALRGSFLLDAD	21
$AhpC_{134-143}$	TVRHAVVNDL	10
$AhpC_{4-16}$	TKKALDFTAPAVL	13
$AhpC_{166-172}$	GEVCPAG	7
$AhpC_{68-73}$	EVIGIS	6

3.2. Expression and purification of AhpC protein

To obtain full length protein of AhpC, we cloned the sequence into pGEX-4T-1. After sequencing, the peak shape appeared to be normal and the target gene was synthesized correctly. After purification and expression of the recombinant protein by SDS-PAGE electrophoresis (Fig. 1A) and western blot (GST-tagged antibody, Fig. 1B), the results revealed that there was a single band at 48 kDa, indicating that the protein was purified correctly and with high purity.

3.3. Antigenicity analysis of designed epitopes

To clarify the potential antigenicity of these epitopes, we immunized three healthy male rabbits following the animal immunization process, and purified the antibodies from them separately. Dot blot analysis revealed that in both antibodies 1 and 2, peptide $AhpC_{4-16}$ demonstrated dominant cross-reactivity compared to the other six epitopes (Fig. 2A). In the antibody 3, peptide $AhpC_{112-132}$ and $AhpC_{4-16}$ showed higher ability to cross-react. This result was confirmed by two other individual experiments.

For further analysis of antigenicity, we diluted purified antibodies with gradient concentrations (1:200, 1:1000, 1:5000, 1:10,000, 1:20,000, 1:60,000, 1:240,000) and tested by ELISA. Antibody 1 showed strong cross-reactivity with peptides $AhpC_{4-16}$ and $AhpC_{34-57}$ when diluted by 200 and 1000 folds. When diluted the antibody (5000 fold), only peptide $AhpC_{4-16}$ has the ability when compared to others (Fig. 2B). Besides, peptide $AhpC_{4-16}$ displayed the highest potential ability cross-reaction in various dilutions of the antibody 2 (Fig. 2C). Although the situation was more complicated about antibody 3, epitope $AhpC_{4-16}$ demonstrated dominant cross-reactive ability in 1000 and 5000 fold dilutions (Fig. 2D). In conclusion, all these results suggest that peptide $AhpC_{4-16}$ was the dominant AhpC B cell epitope.

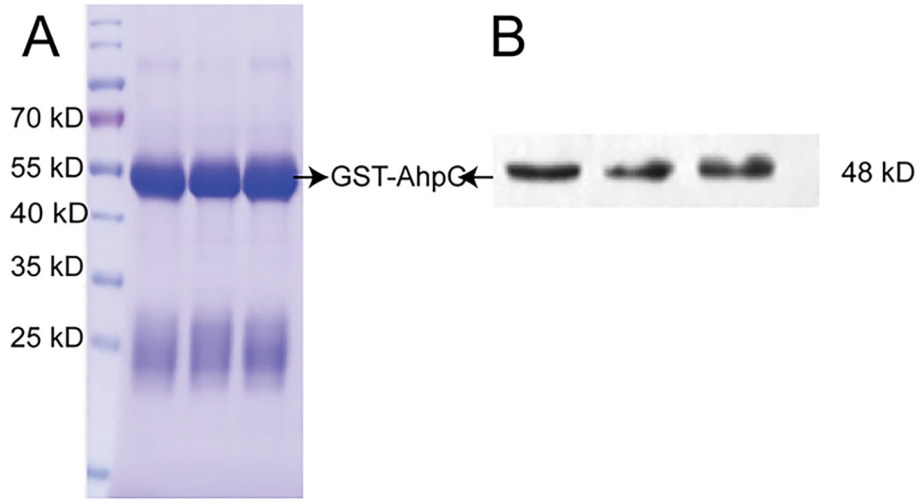

Fig. 1. SDS-PAGE electrophoresis and western blot of GST-AphC recombinant protein. A is SDS-PAGE and B is GST-tagged antibody western blot.

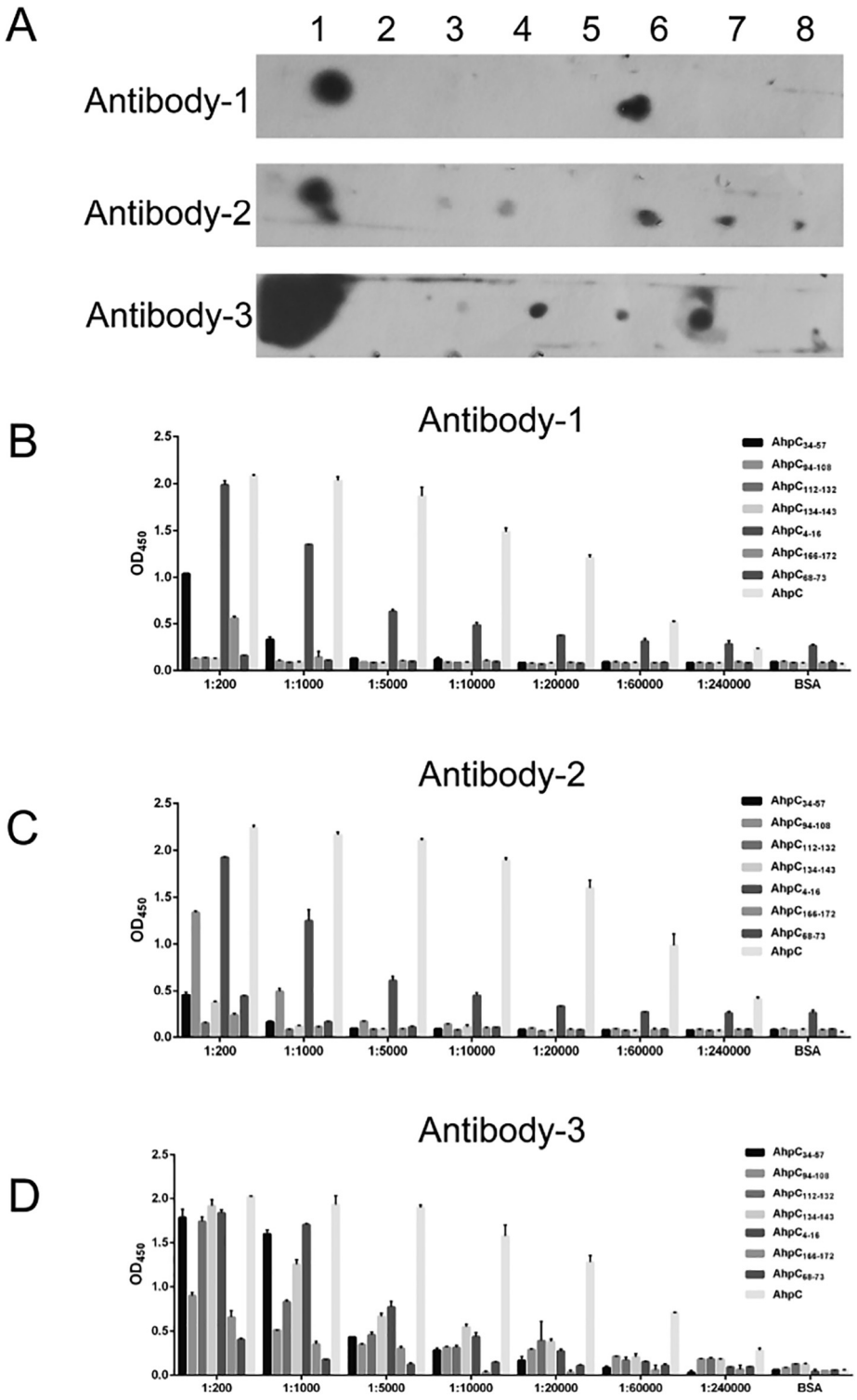

Fig. 2. Specificity and sensitivity of synthesized short AhpC epitopes. A: Dot blot results of the purified antibody and AhpC protein, polypeptide. 1 is AhpC, 2 is $AhpC_{34-57}$, 3 is $AhpC_{94-108}$, 4 is $AhpC_{112-132}$, 5 is $AhpC_{134-143}$, 6 is $AhpC_{4-16}$, 7 is $AhpC_{166-172}$, 8 is $AhpC_{68-73}$. B-D: The reaction of three purified antibodies with peptides and AhpC protein was detected by ELISA.

3.4. Immunogenic potential of the dominant epitope in immunized mice

We proved that epitope $AhpC_{4-16}$ was the dominant B cell epitope of protein AhpC, but whether it could induce immune response *in vivo* was still unknown. To explore this issue, serum from immunized mouse was collected two weeks after the final immunization. Immune response generated by peptide $AhpC_{4-16}$ or AhpC was examined by serum IgG levels. The amount of IgG in peptide $AhpC_{4-16}$ immunized mice was significantly higher when compared to the control group, and the level was similar to full length protein AhpC immunized group (Fig. 3A). In addition, the cells from the spleens were isolated from immunized mice and cultured with *C. jejuni* for 24 h. The levels of IL-4 were much higher

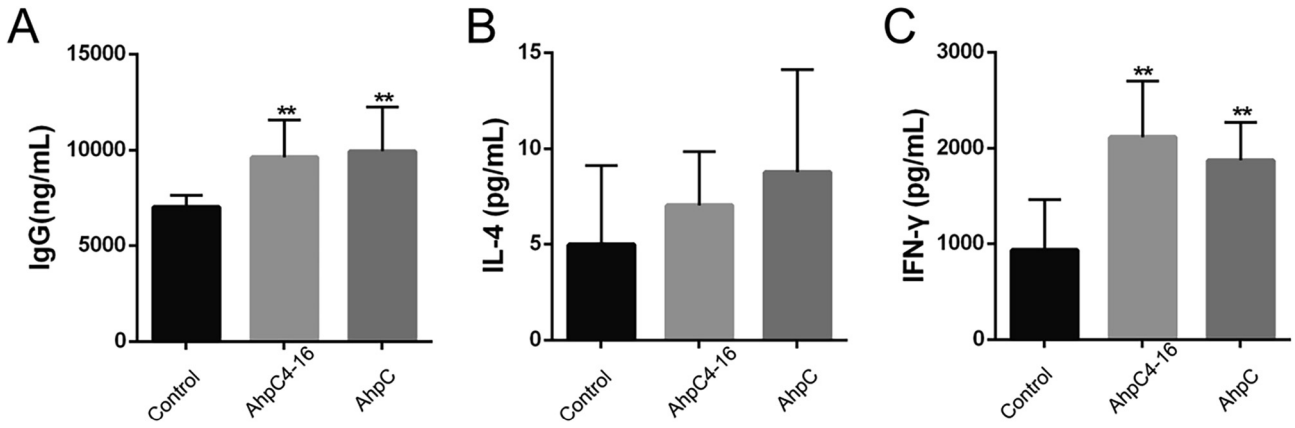

Fig. 3. Immune response caused by immunization. A: Expression levels of IgG in serum after immunization were determined by ELISA assay. B: The ability of peptide $AhpC_{4-16}$ and protein AhpC on inducing the immune response displayed by changes of IL-4 expression in cocultured medium of isolated spleen cells and *C. jejuni*. C: IFN-γ was detected in the same conditions as described above. $^{**}P < 0.01$ vs. control.

in peptide $AhpC_{4-16}$ immunized group than the control group, but showed no significant difference between them (Fig. 3B). However, the expression of IFN-γ was induced more in the peptide $AhpC_{4-16}$ immunized group compared to control group (Fig. 3C). This indicated that the dominant epitope $AhpC_{4-16}$ can induce immune response in mice and may display protective role after infection.

3.5. Protective role of the dominant epitope

To determine whether the dominant epitope has protective role against *C. jejuni* infection *in vivo*, mice were injected with *C. jejuni* four weeks after the final immunization, with an amount of 5×10^9 CFU for each injection. Illness information (health: 0, illness: 1, death: 2) was recorded until 2 weeks post challenge. The illness index and protective rate were shown in Table 2.

Histology was done on 10 mice of each group. These changes are classified as mild when there was a light inflammatory infiltration without destructive changes (Fig. 4A), which was mostly observed in the $AhpC_{4-16}$ immunized group and AhpC immunized group. Inflammatory exudate in the lumen and partial necrosis of luminal and mucosal were defined as moderate inflammation (Fig. 4B). Tissues were classified with severe inflammation when displayed subtotal peeling off mucosa, with significant inflammatory infiltration, necrosis as well as lumen exudate (Fig. 4C), which was mostly observed in the control group. The pathological changes in jejunal tissues of different groups are summarized in Table 3.

Table 2
The illness index and protective rate of the three groups (means ± SD, n = 10).

Groups	Illness index (score)	Protective rate (%)
control	1.20 ± 0.37	0
$AhpC_{4-16}$	0.40 ± 0.25*	66.67
AhpC	0.20 ± 0.20*	83.33

* $P < 0.05$ vs. control.

Table 3
Summary of the pathological changes in the jejunal tissues of the three groups (n = 10).

Groups	Mild	Moderate	Severe
Control	1	3	6
$AhpC_{4-16}$	5	5	0
AhpC	7	3	0

4. Discussion

C. jejuni is the main cause of campylobacteriosis, and is still one of the most common bacteria that cause intestinal diseases worldwide (DuPont, 2009). Vaccine is an efficient and economical way to protect our bodies against various bacterial infections. However, *C. jejuni* vaccine has not received much progress due to differences in molecular structure of different serotype antigens, and potential peripheral nerve immunological damage (Maue et al., 2014; O'Ryan et al., 2015). Moreover, with the increasing number of resistant strains of *C. jejuni*, it is of great practical significance to seek a new, safe and effective subunit vaccine of *C. jejuni* (Shin et al., 2015; Mäesaar et al., 2016). Many methods have been explored to develop new vaccines. B-cell epitope related immunogens have also been evaluated widely due to their vaccine potential

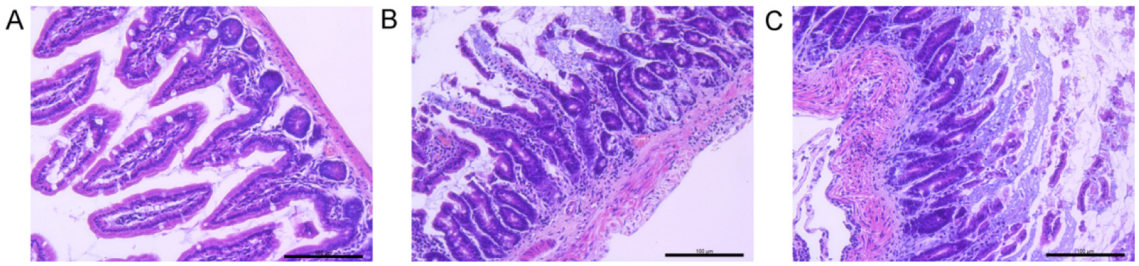

Fig. 4. Histopathological staining of the mice jejunum tissue challenged with *C. jejuni*. A: Mild inflammation: intact mucosa, minimal inflammation, and a mild inflammatory infiltrate without mucosal destruction. B: Moderate inflammation: partially denuded mucosa, fibrinous exudates in the lumen, with mixed inflammatory infiltrate. C: Severe inflammation: denuded mucosa, prominent inflammatory infiltrate, necrosis and luminal exudates, scale bar 100 μm.

for numerous pathogens. Evaluating the dominant epitopes of B-cell epitope helps people to understand the mechanisms involved in the protective responses, and will also help us to design the vaccine in a more efficient manner (Yasmin et al., 2016). In this study, we used Immune Epitope Database Analysis Resource to predict B cell epitope.

AhpC has been explored in developing vaccines for many diseases (O'Riordan et al., 2012). In our research, seven peptide sequences of AhpC were designed, and AhpC recombinant protein was purified and expressed through pGEX-4T-1 plasmid system. We first evaluated these seven B-cell epitopes of AhpC by testing their cross-reacting capacity by dot blot or ELISA methods. Our study results also showed the most sensitive reactions when evaluated this with various dilutions of the antibody obtained from AhpC immunized rabbits. These results indicated that epitope $AhpC_{4-16}$ was the dominant B cell epitope of AhpC in *C. jejuni*. To further determine its vaccine potential, we immunized the mice with dominant peptide or full length AhpC. It is well known that when a whole protein antigen provokes an immune response, the active ingredient then acts as the dominant epitope (Akram and Inman, 2012). In the vaccine response of *C. jejuni*, antibody response plays a major protective role (Shin et al., 2015), especially humoral response remains to be important (Lee et al., 1999; Sizemore et al., 2006). In this study, IgG of the serum was obviously induced in peptide $AhpC_{4-16}$ immunized group and AhpC immunized group, and the differences in the expression levels were significant compared to the control group. Isolated cells from immunized spleen also displayed the ability of protection when cultured with *C. jejuni* for 24 h, as the expression levels of IL-4 and IFN-γ were both higher in peptide $AhpC_{4-16}$ immunized group than the control group. This confirmed that the inoculation of dominant B cell epitopes significantly enhanced humoral responses in mice.

To further demonstrate the possibility that peptide $AhpC_{4-16}$ can be used as a vaccine against *C. jejuni in vivo*, we evaluated its protective role against *C. jejuni* infections. When attacked by *C. jejuni* (5×10^9 CFU), mice in the control group exhibited prominent clinical signs including inactivity, lack of responsiveness to stimulation, reduced eating or drinking, hunched posture, ruffled hair coat, soft feces or diarrhea. Histological observation of the ileum showed partially denuded mucosa, fibrinous exudates in the lumen, with mixed inflammatory infiltrate. These phenomena confirmed that the mice had campylobacteriosis as described (Jain et al., 2008; Mihaljevic et al., 2007). In peptide $AhpC_{4-16}$ immunized group and AhpC immunized group, the illness index was significantly lower and the protective rates were increased significantly. These evidences indicated that epitope $AhpC_{4-16}$ indeed protected the mice from *C. jejuni* infections as it can rescue the disorder observed in the control mice.

5. Conclusion

We identified the dominant B cell epitope as $AhpC_{4-16}$ from seven predicted candidates. It not only showed high sensitivity for cross-reactivity, but also displayed strong ability to induce immune responses *in vivo*. Apart from this, *C. jejuni* infected mice acquired benefits from peptide $AhpC_{4-16}$ immunization as its jejunum showed less disorder compared to control mice, indicating epitope $AhpC_{4-16}$ as a candidate epitope of MAP vaccine against this pathogen.

Acknowledgements

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Declaration of Competing Interest

The authors declare no conflicts of interest.

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