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PRODUCTION OF POLYCLONAL ANTIBODY AGAINST THE OUTER MEMBRANE PROTEIN Omp48 OF *Aeromonas hydrophila*

Thi Hoa Rol[1], Nguyen Van Hau[1], Do Nguyen Trong Tri[1], Dang Thi Phuong Thao[1,2], Nguyen Thi My Trinh[1,2]*

Address(es):
[1]Department of Molecular and Environmental Biotechnology, University of Science, Ho Chi Minh City, Vietnam.
[2]Vietnam National University, Ho Chi Minh City, Vietnam.

*Corresponding author: ntmtrinh@hcmus.edu.vn
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ABSTRACT

Aeromonas hydrophila is one of the main pathogens in fish, causing a huge economic loss every year. Since outer membrane protein Omp48 had been demonstrated to be immunogenic in fish, it has gained attention as a potential vaccine candidate to protect fish from *A. hydrophila*. In this study, we produced a polyclonal antibody against Omp48, which would be necessary for further studies on this protein. To reach this aim, 6xHis-tagged Omp48 was expressed in a recombinant *E. coli* strain and purified by a nickel affinity chromatography column, which yielded a purity of 97.3 ± 1.4%. The purified Omp48 was injected into rabbits in order to produce polyclonal anti-Omp48 antibody. Subsequently, we analyzed the obtained antiserum, which was demonstrated to have high specificity and sensitivity to the Omp48 protein by Western blot and ELISA, with the titer of 1:1,280,000. In addition, we also showed that the antiserum could be used to detect Omp48 in immunofluorescence staining.

Keywords: *A. hydrophila*, Outer membrane protein, Omp48, polyclonal antibody

INTRODUCTION

Fish farming has a long history dating to ancient China but only started to explosively expand and significantly contribute to the food supply about 30 years ago. According to Food and Agriculture Organization (FAO) **(2020)**, farmed fish production has increased 6 times from 1990 to 2018, making it the fastest-growing food-production industry in the world. In 2018, it provided estimately 54 million tonnes of products worldwide, generating an income of USD 250 billion **(FAO, 2018; FAO, 2020)**. However, fish farming is usually threatened by infectious diseases outbreaks. The main reason is that intensive culture systems, which are commonly used to maintain a large number of fish in a small area, provide the ideal conditions for the growth and spread of pathogens. Additionally, in crowded and unnatural environments, fish are stressed and more vulnerable to diseases. Therefore, diseases frequently occur in fish farming all over the world, resulting in an estimated economic loss of USD 1.05 to USD 9.58 billion/year **(Tavares-Dias & Martins, 2017)**.

Aeromonas hydrophila is a major pathogen which is responsible for various outbreaks in fish farming. *A. hydrophila* is a motile, Gram-negative, nonspore-forming, rod-shaped bacteria which belongs to the family Vibroonaceae **(Ünüvar, 2018).** This bacteria is associated with acute hemorrhagic septicemia and diffuse necrosis in carp, salmon, goby, catfish, and dogfish, causing a very high mortality rate (80-100%) in a short time (within 1-2 weeks) **(Rosidah *et al.*, 2019)**. It was reported that *A. hydrophila* outbreaks were responsible for a yield reduction of 2,200 tonnes of fish per year between 1989-1991 in Zhejiang, China, and an economic loss of more than USD 12 million in the southeastern United States in 2009 **(Hossain *et al.*, 2014; Nielsen *et al.*, 2001)**. Therefore, controlling the infection of *A. hydrophila* is critical for successful fish farming.

Recently, bacterial outer membrane proteins (OMPs) have gained attention as potential candidates for vaccine development due to their high immunogenicity and high conservation within Gram-negative bacteria. In addition, various OMPs contain pathogen-associated molecular patterns, which can be recognized by immune cells such as monocytes, macrophages, neutrophils, and dendritic cells **(Maiti *et al.*, 2020)**. Rahman and Kawai **(2000)** reported that the mortality rate of goldfish challenged with *A. hydrophila* could be significantly reduced by pre-immunization with OMPs extracted from this pathogen.

Omp48 is one of the *A. hydrophila* outer membrane proteins that have been clearly demonstrated to be immunogenic in fish. Khushiramani *et al.* reported that the immunization with recombinant Omp48 significantly increased the survival of fish challenged with *A. hydrophila* and *E. tarda*, suggesting the potential of Omp48 as a vaccine candidate against these two pathogens **(Khushiramani *et al.*, 2012)**. However, further studies on the characteristics and applications of Omp48 require a specific antibody, which has not been available yet. In this study, we succeeded in producing a polyclonal antibody against Omp48 and demonstrated that the obtained antibody could be used to detect this protein in various assays, including ELISA, Western Blot, and immunofluorescence analysis.

MATERIAL AND METHODS

Plamids and strains

The *A. hydrophila* stain was cultured in tryptone broth medium and its genome was isolated using a protocol previously described by Hiney *et al.* (**1992**). The gene encoding Omp48 was amplified from *A. hydrophila* genome by PCR using primer pair AGAAGGAGATATACCATGGTTGATTTCCACGGCTACTTCC and TTGTTAGCAGCCGGATCTCAGTGGTGGTGGTGGTGGTGCTCGAGCCACCAAGCTTCCGC. The amplicon was cloned into *Nco*I/*Xho*I sites of plasmid pET-28a(+) (Novagen) in-frame with polyhistidine (6xHis) tag sequence using eClone cloning kit (Molecular Biotech Lab., VNUHCM-University of Science, Vietnam) to construct pET-omp48. The cloned product was sequenced using Sanger method and the sequence of *omp48* was then verified using nucleotide BLAST tool (https://blast.ncbi.nlm.nih.gov). After that, the plasmid pET-omp48 was transformed into BL21(DE3) cells to generate BL21(DE3)/pET-omp48 strain. The empty plasmid pET-28a(+) was also introduced into BL21(DE3) cells to generate a negative control strain BL21(DE3)/pET-28a(+). The *A. hydrophila* strain used in this study was isolated and provided by Research Institute for Aquaculture No. 2, Ho Chi Minh City, Vietnam.

Omp48 expression and purification

BL21(DE3)/pET-omp48 cells were cultured in an Erlenmeyer flask (1 L) containing 300 ml LB medium (1% tryptone, 0.5% yeast extract, and 0.5% sodium chloride) supplemented with 50 µg/ml kanamycine at 37 °C with shaking at 240 rpm. When the culture reached an OD_{600} of 0.81.0, isopropyl β-d-1-thiogalactopyranoside (IPTG) was added at the final concentration of 0.5 mM and cells were further cultured at 37 °C for 4 hours. After that, cells were collected by centrifugation at 10,000 rpm, 4 °C for 5 minutes. Cells were then resuspended in lysis buffer containing 50 mM Tris-HCl pH 8 and 1 mM EDTA with the ratio of 10 ml buffer per gram crude cell pellet (wet weight) and disrupted using M-110EH-30 Microfluidizer Processor. The cell lysate was then centrifuged at 13,000 rpm, 4 °C for 10 minutes to separate soluble and insoluble fractions. The insoluble fraction was dissolved into binding buffer (20 mM PBS, 8 M urea, and 10 mM imidazole, pH 8) with the ratio of 20 ml buffer per gram pellet (wet weight) at 4 °C for 16

hours. After that, the solution was centrifuged at 13,000 rpm, 4 °C for 30 minutes to remove all insoluble particles before being used for Omp48 purification.
Since Omp48 was fused with a 6xHis-tag at its C-terminus, a 5 ml HisTrap FF column (GE Healthcare, US) was used for the purification of Omp48. Firstly, the column was equilibrated with 25 ml binding buffer (20 mM PBS, 8 M urea, and 10 mM imidazole, pH 8). After that, all of the Omp48 dissolved solution was applied into the column. The column was then washed with 25 ml washing buffer (20 mM PBS, 8 M urea, and 40 mM imidazole, pH 8). Finally, the target protein Omp48 was eluted using elution buffer (20 mM PBS, 8 M urea, and 250 mM imidazole, pH 8). All steps were performed at a flow velocity of 2 ml/min. All fractions including flow-through, washing and elution were collected for SDS-PAGE analysis. The purified Omp48 was overnight dialyzed into 10 mM phosphate buffer saline (PBS) pH 7.4 at 4 °C prior to immunization.
For SDS-PAGE analysis, 10 µl of each sample was applied into each well of a 15% polyacrylamide gel. Gels were stained using a standard Coomassie blue staining method **(Brunelle & Green, 2014)** or silver staining method **(Kavran & Leahy, 2014)**. The purity of protein was determined by densitometry of silver-stained SDS-PAGE gel using Gel Analyzer software (www. gelanalyzer.com). For Western Blot analysis, a monoclonal anti-Hisdidine antibody (H-3, SC 8036, Santa Cruz Biotechnology) and an anti-mouse IgG, HRP-link antibody (Sigma) were used as primary and secondary antibodies to detect 6xHis-tagged Omp48. The concentration of protein was determined using Bradford protein assay kit (Bio-rad, US).

Immunization of rabbits with Omp48

Two healthy female New Zealand white rabbits (~2.5 kg, 10-15 weeks of age) were purchased from Pasteur Institute, Ho Chi Minh City, Vietnam. Pre-immune sera were collected from rabbits before immunization for negative controls. For the primary immunization, 50 µg purified Omp48 was mixed with an equal volume of complete Freund's adjuvant and subcutaneously injected into rabbits. For booster immunizations, 50 µg purified Omp48 was mixed with an equal volume of incomplete Freund's adjuvant and subcutaneously administered four times every two weeks later. One week after the last immunization, sera were collected, mixed, and stored at -80 °C for further use.

Evaluation of Omp48 immunized serum

The presence and specificity of anti-Omp48 antibody in immunized serum were examined by Western Blot, indirect enzyme-linked immunosorbent assay (indirect ELISA), and immunofluorescence staining.
For Western Blot, 20 µg of total protein from *E. coli* or *A. hydrophila* cells, or 10 µg of purified Omp48 was loaded into each well of a 15% polyacrylamide gel. The obtained antiserum at the dilution of 1:10,000 and an anti-rabbit IgG, HRP-link antibody (Invitrogen) at the dilution of 1:20,000 was used as primary and secondary antibodies, respectively. The signals were visualized using SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific).
For indirect ELISA, 100 µl of recombinant Omp48 in coating buffer (0.1 M Na_2CO_3, 0.1 M $NaHCO_3$, 0.04g sodium azide, pH 9.4) at desired concentration was added into each well of a 96-well microtiter plate. The plate was incubated at 37 °C for 3 hours. After that, the protein solution was removed and 100 µl of blocking buffer (5% skim milk in 10 mM PBS pH 7.4) was added into each well. The plate was incubated at 25 °C for at least 1 hour. After blocking step, the collected antiserum was 2-fold serially diluted in PBS from 1:10,000 to 1:2,560,000 and 100 µl of antiserum at each dilution was added into each well. The plate was incubated at 25 °C for 1 hour. Then, 100 µl of the anti-rabbit IgG, HRP-linked antibody (Invitrogen) at the dilution of 1:10,000 was added into each well. After antibodies incubation, 100 µl of 1-Step™ Ultra TMB-ELISA Substrate Solution (ThermoFisher Scientific) was added into each well and the plate was incubated at 25 °C for 15 minutes. The reaction was then stopped by adding 100 µl of 2N H_2SO_4 solution into each well. The result was read at 450 nm wavelength by Multiskan Ascent microplate. A similar experiment using pre-immunized serum instead of Omp48-immunized serum was concurrently performed as negative controls. The antibody titer was defined as the highest antiserum dilution when its OD_{450} value was at least 2 folds higher than that of the negative control.
For immunofluorescence staining, *A. hydrophila* cells were cultured at 37 °C with shaking in Tryptic soy broth (HIMEDIA, India). When the culture reached OD_{600}=0.5-0.8, cells were collected by centrifugation. Cells were then incubated with 1% BSA in PBS for 30 min at 25 °C to block unspecific binding of the antibody. After that, cells were incubated with the diluted antiserum in PBS (1:1000) at 25 °C for 2 hours. Finally, cells were incubated with diluted anti-rabbit fluorescence Alexa 488 antibody (Invitrogen) in PBS (1:500) at 25 °C for 2 hours. All samples were observed without fixation using An Eclipse Ni-U Upright microscopic system (Nikon) at the same exposure time.

RESULTS AND DISCUSSION

Expression and purification of 6xHis-tagged Omp48

To induce the expression of 6xHis-tagged Omp48, 0.5 mM IPTG was added to the exponentially growing culture of *E. coli* BL21(DE3)/pET-omp48. Four hours later, cells were collected and the expression of Omp48 was verified using SDS-PAGE and Western Blot analyses.
We found that there was an intense band around 48 kDa present in the IPTG-treated BL21(DE3)/pET-omp48 sample (Fig 1A, well 4) but absent in all negative control samples (Fig 1A, wells 1-3). Importantly, this band was detected in Western Blot analysis using an anti-Histidine antibody (Fig 1B, well 4). These results clearly indicated the overexpression of Omp48 in the recombinant *E. coli* cells. In addition, we also found that Omp48 was mostly expressed in inclusion bodies since the same protein band was observed in the insoluble fraction of cell lysate (Fig 1A-B, well 5) but not in the soluble fraction (Fig 1A-B, well 6).

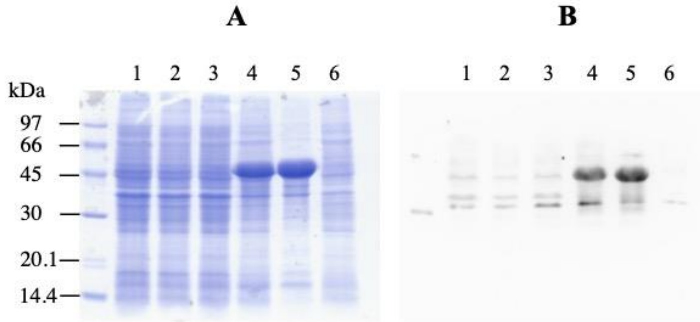

Figure 1 The expression of 6xHis-tagged Omp48 in *E. coli* BL21(DE3)/pET-omp48. The presence of Omp48 was verified by SDS-PAGE (A) and Western Blot analysis using an anti-Histidine antibody (B). Samples were as follows:1, IPTG-untreated BL21(DE3)/pET-28a(+); 2, IPTG-treated BL21(DE3)/pET-28a(+); 3, IPTG-untreated BL21(DE3)/pET-Omp48; 4, IPTG-treated BL21(DE3)/ pET-Omp48, total protein; 5, IPTG-treated BL21(DE3)/ pET-Omp48, insoluble fraction; 6, IPTG-treated BL21(DE3)/ pET-Omp48, soluble fraction.

Therefore, in the next step, we dissolved inclusion bodies in 8M urea buffer, and then the dissolved fraction was applied to Ni-NTA affinity chromatography for purifying Omp48 under denaturing condition. The SDS-PAGE analysis of protein purification showed that the majority of contaminant proteins were observed in the flow-through fraction (Fig 2, well 2) and thus, the target protein Omp48 was obtained with high purity (97.3 ± 1.4%) and high recovery (78.8 ± 5.5%) in the elution fraction (Fig 2, well 4). The purified Omp48 was then dialyzed in phosphate buffer to remove urea and imidazole for the injection into rabbits in order to produce Omp48 antibody.

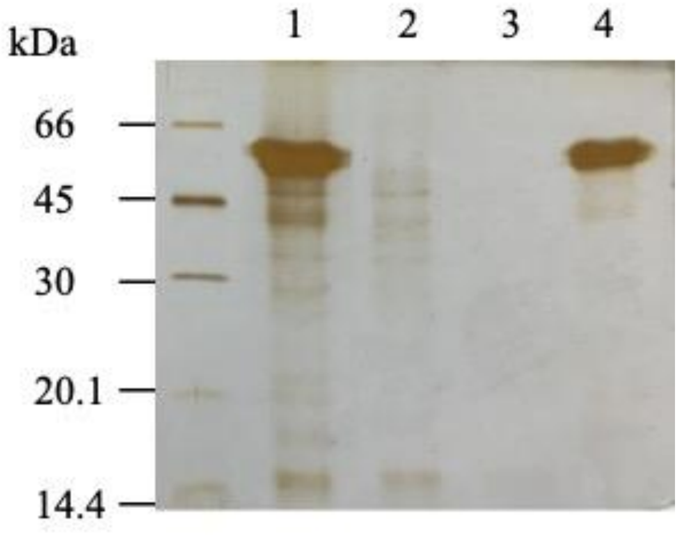

Figure 2 The result of Omp48 purification. Samples were as follows: 1, pre-purified Omp48; 2, flow-through fraction; 3, washing fraction; 4, elution fraction.

The production of polyclonal anti-Omp48 antibody and its verification by Western Blot analysis

The Omp48 polyclonal antibody was produced using a 90-day rabbit immunization protocol. The presence of Omp48 antibody in the serum was demonstrated by Western Blot analysis, in which a clear signal around 48 kDa was observed in Omp48 containing samples, including IPTG-treated BL21(DE3)/pET-omp48, purified Omp48, and *A. hydrophila* samples (Fig 3, wells 2-4). Notably, no bands were detected in BL21(DE3)/pET-28a(+) sample (Fig 3, well 1). These results indicated that the obtained antiserum can be used to detect both the recombinant and the native Omp48 in *A. hydrophila* cells.

We also found another band which was lower than that of Omp48 in the Western Blot result of *A. hydrophila* sample (Fig 3B, well 4). Our analysis using protein BLAST on NCBI showed that a 46 kDa maltoprotein derived from *A. hydrophila* (Genbank No: WP_039212957.1) matches 57% with the target Omp48 sequence. Although the protein corresponding to the lower band has not been identified, the result of BLAST analysis suggests that *A. hydrophila* might express a protein sharing high similarity with Omp48, and thus, this protein can also be detected by the obtained antiserum.

In addition, when verifying the expression of 6xHis-tagged Omp48 in *E. coli* by Western Blot analysis with anti-histidine antibody, we found a faint signal at the same position as Omp48 in the negative controls (Fig 1B, wells 1-3). We suspect that *E. coli* cells might contain a histidine-rich protein having the same molecular weight as Omp48. Therefore, this protein was also recognized by the anti-histidine antibody. However, the absence of this band in the BL21(DE3)/pET-28a(+) sample in Western Blot result using the obtained antiserum (Fig 3B, well 1) indicated that the histidine-rich protein mentioned above did not affect the specificity of anti-Omp48 antiserum.

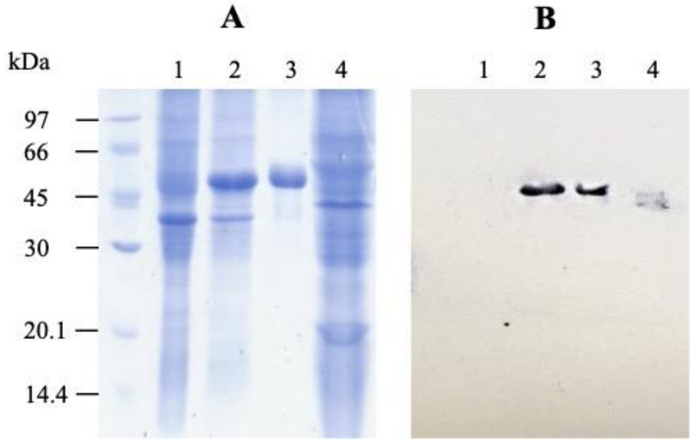

Figure 3 The verification of anti-Omp48 antibody by Western Blot analysis. Samples, including protein lysates from BL21(DE3)/pET-28a(+) cells (1), IPTG-treated BL21(DE3)/pET-Omp48 cells (2), purified Omp48 (3), and *A. hydrophila* cells (4), were analyzed by SDS-PAGE (A) and Western Blot with the obtained antiserum at the dilution of 1:10,000 (B).

Evaluation of antibody affinity by ELISA

The sensitivity of anti-Omp48 antibody was evaluated by indirect ELISA method, in which the purified recombinant Omp48 at concentrations of 0.39-200 ng/ml was coated into wells of a microtiter plate and then incubated with the obtained antiserum at the dilution of 1:10,000. The similar wells incubated with pre-immunized serum were concurrently prepared for negative controls. The results in Fig. 4A showed that no reactivity between pre-immunized serum and Omp48 was detected ($OD_{450} < 0.1$). Upon incubation with Omp48-immunized antiserum, the OD_{450} values reached maximal level (~2.0) in the well coated with 200 ng/ml Omp48 and gradually decreased when the lower concentrations of Omp48 were used. However, even at the lowest tested concentration of Omp48 (0.39 ng/ml), the OD_{450} value was still significantly higher than that of the well incubated with the pre-immunized serum, indicating that the obtained antiserum at the dilution of 1:10,000 can be used to detect Omp48 at the concentration of 0.39 ng/ml or higher. In addition, when incubating Omp48 at the concentration of 100 ng/ml with the obtained antiserum at different dilution rates from 1:10,000 to 1:2,650,000, we found that the OD_{450} values of the antiserum incubated wells were remarkable higher than those of the negative controls at the dilution of 1:1,280,000 and higher, but there was no significant difference between the OD_{450} values of wells incubated with antiserum and pre-immunized serum at the dilution of 1:2,560,000 (Fig. 4B), suggesting that 1:1,280,000 is the titer of the obtained antiserum for the detection of Omp48 at the concentration of 100 ng/ml in ELISA assay.

The produced Omp48 antibody can be used for immunostaining

The immunostaining assay was performed on *A. hydrophila* cells using the Omp48-immunized serum at the dilution of 1:1000. The negative control was performed using a similar protocol but with the pre-immunized serum instead of Omp48-immunized serum. The results in Fig. 5 showed that clear signals were observed in cells incubated with the anti-Omp48 antiserum but not in the negative sample. These data clearly suggest that the serum obtained from this study can be used for immunostaining.

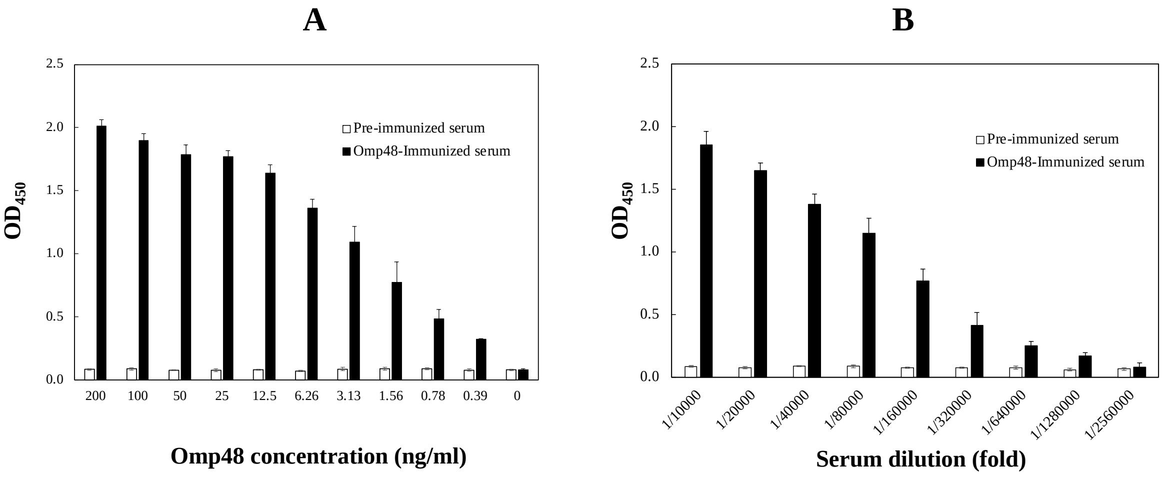

Figure 4 Evaluation of anti-Omp48 antibody by ELISA. A. the purified Omp48 at different concentrations was coated into each well of a microtiter plate and incubated with the pre-immunized and Omp48-immunized sera at the dilution of 1:10,000; B. the purified Omp48 at the concentration of 100 ng/ml was coated into each well and incubated with the pre-immunized and Omp48-immunized at different dilution rates. Data were shown as mean ± SD of three repeats.

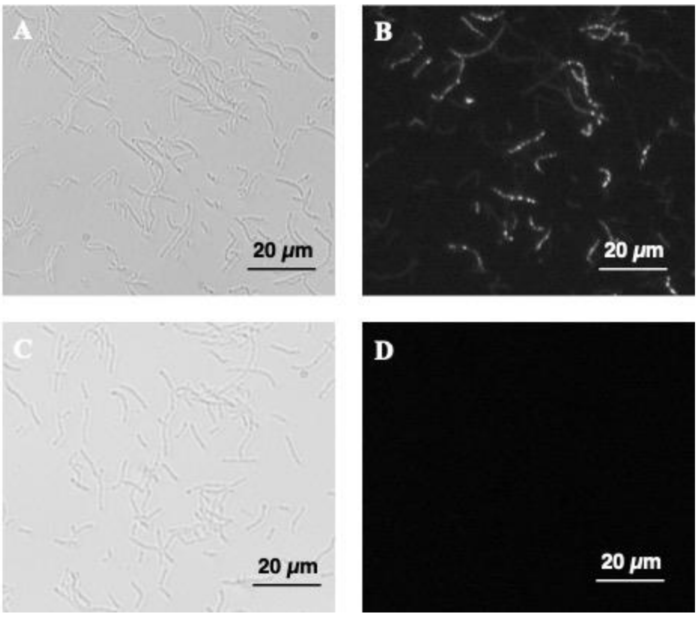

Figure 5 Immunostaining analysis of *A. hydrophila* cells. Cells were stained with Omp48-immunized serum (A, B) or pre-immunized serum (C, D) at the dilution of 1:1000. The bright-field (A, C) and fluorescent images (B, D) were captured using a fluorescence microscopic system.

CONCLUSION

In this study, we succeeded in producing a specific polyclonal antibody against the outer membrane protein Omp48 of *A. hydrophila.* The antigen was generated by overexpressing 6xHis-tagged Omp48 in *E. coli* cells. After purification using Ni-NTA affinity chromatography, the recombinant Omp48 with a purity of 98.53% was injected into rabbits to produce polyclonal antibody. The obtained antiserum had a high specificity and sensitivity for detection of Omp48 in Western Blot and ELISA assays, with a titer of 1:1,280,000. We also demonstrated that the antiserum can be used for immunofluorescence staining. Although the raw antiserum can be directly used for these assays, the purification of antibodies from serum might be needed for further purposes, such as labelling them with an enzyme or a fluorescent-tag. The availability of the Omp48 antibody would facilitate studies on *A. hydrophila* Omp48.

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