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PRODUCTION AND EVALUATION OF AN ANTISERUM AGAINST ENVELOPE PROTEIN VP28 OF WHITE SPOT SYNDROME VIRUS

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SUMMARY

Virus-associated white spot syndrome (WSSV) is one of the most popular diseases in shrimp, causing a huge economic loss globally and also in Vietnam. Protein VP28 plays an important role in the initial steps of WSSV infection into shrimp cells. This protein binds to the shrimp cells as a viral adhesion molecule and then helps the virus enter the host cells. Therefore, many studies and applications related to the control of this virus have targeted VP28 as a potential antigen. To facilitate the study and application of VP28, this study focused on the development of a VP28-specific antiserum. The *E. coli* BL21(DE3)/pQE30-*vp28* cells were cultured in LB medium supplemented with 100 µg/mL ampicillin and 0.5 mM IPTG was added to induce the expression of VP28. The cells were then disrupted by sonication and the supernatant fraction was used to purify VP28 using Ni-NTA affinity chromatography. The purified VP28 was injected into rabbits following a 90-day immunization protocol. The serum was then collected and validated for the ability to detect VP28 in Western Blot and ELISA. We found that the obtained antiserum could bind to both the recombinant and native VP28 in Western Blot assay. We also demonstrated that the antiserum could recognize VP28 in ELISA with the sensitivity of <0.39 ng/wells VP28 and the titer of 1/1,280,000. We successfully produced an anti-VP28 antiserum which can be used as a primary antibody in Western Blot and ELISA.

Keywords: VP28, White Spot Syndrome Virus, antiserum, Western Blot, ELISA

INTRODUCTION

Shrimp farming is one of the biggest agricultural industries in Vietnam with the total yield of 745 thousand tons and the export turnover of 4.1 billion USD in 2022 (Hương, 2023). However, the shrimp farming is frequently in risk of infectious diseases, that challenges the improvement of this industry. White spot syndrome virus is

among the most popular and serious pathogens with the fatality rate of 90-100%. In Vietnam, the outbreak of WSSV was first reported in the Southern area in 1994-1995 and quickly spread to other farms all over the country. According to Vietnam Department of Animal Health, in 2022, this disease occurred in 177 villages of 19 provinces, from Quang Ninh to Ca Mau, and affected 2,374 ha of farming area (Huệ, 2022).

WSSV is an enveloped, rod-shape virus, typically 70-150 nm in diameter and 250 – 380 nm in length (Xie *et al.*, 2015). It has a double-stranded DNA genome with the size of approximately 290 kb with 184 predicted coding genes (Tang *et al.*, 2007; van Hulten *et al.*, 2000). Among proteins expressed by WSSV genome, the envelope protein VP28 has gained attention due to its high potential application in vaccine development. According to Witteveldt *et al.* (2004), VP28-based vaccine could protect shrimps from WSSV infection. Solís-Lucero *et al.* (2016) reported that the injection with phage-displayed-VP28 vaccine reduced the mortality of WSSV-challenged shrimps. Recently, Linh *et al.* (2021) also reported that the feeding with VP28-anchored yeast cells provided a good protection to shrimps against WSSV with a relative percent survival of 87.10 ± 2.15%.

Thus, this study was carried out in an attempt to produce an anti-VP28 antiserum, which can be used as a primary antibody to detect VP28 in Western Blot, ELISA, and immunofluorescence staining for VP28-based vaccine development or for other studies on this protein in Vietnam. Accordingly, the recombinant VP28 was expressed in *E. coli* cells and then obtained for the immunization in rabbits. The antisera from rabbits were collected and evaluated for the reactivity with VP28 in Western Blot and ELISA.

MATERIALS AND METHODS

Construction of an *E. coli* strain expressing recombinant VP28

The *E. coli* strain BL21(DE3)/pQE30-*vp28* capable of expressing recombinant VP28 was previously constructed by Trang *et al.* (2003). The gene *vp28* was obtained from WSSV genome by PCR and inserted into the vector pQE-30 (Qiagen) at *Bam*HI/*Sal*I sites to create plasmid pQE30-*vp28*. Plasmid pQE30-*vp28* was then introduced into *E. coli* BL21(DE3) cells to establish the *E. coli* BL21(DE3)/pQE30-*vp28* strain.

Expression and purification of VP28

The *E. coli* BL21(DE3)/pQE30-*vp28* strain was inoculated in an Erlenmeyer flask (1 L) containing 300 mL Luria-Bertani medium (10 g/L peptone, 5 g/L yeast extract, and 5 g/L NaCl) supplemented with 100 µg/mL ampicillin (LB-Amp medium) at 37˚C with orbital shaking (250 rpm). When OD_{600} value of the culture reached 0.6 – 0.8, IPTG was added into the medium at the final concentration of 0.5 mM and cells were further inoculated for 4 hours. After that, cells were collected by centrifugation at 6000 rpm and washed once with 100 mM PBS, pH 7.4. Cells were then resuspended in 30 mL buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and disrupted by sonication. The cell lysate was centrifuged at 13,000 rpm, 4˚C for 20 minutes and the supernatant was collected. Since the VP28 protein was fused with a 6xHis tag, the presence of VP28 in collected samples was verified by Western Blot analysis with the 6x-His tag monoclonal antibody (HIS.H8) (Invitrogen, MA1-21315). The goat anti-mouse IgG (H+L)

(HRP, Invitrogen, 62-6520) was used as the secondary antibody.

To purify VP28, the obtained supernatant fraction was applied into a 1 mL Histrap™ HP column at the rate of 0.5 mL/min. The unbound proteins were washed out of the column using washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 7.4). The target protein VP28 was eluted from the column by increasing the ratio of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazole, pH 7.4) up to 100%. All obtained fractions were analyzed by SDS-PAGE and the purity of VP28 was analyzed using Gel analyzer software (www. gelanalyzer.com).

Immunization of rabbits with VP28

The purified VP28 was dialyzed against 100 mM PBS buffer, pH 7.4 at 15˚C for 16 hours using a Spectra/Por™ MWCO 12 – 14 kDa membrane. Protein VP28 was then concentrated using a 10-kDa MWCO centrifugal filer (Millipore) and the final concentration of protein was determined using Bradford method. After that, 1 mL PBS buffer containing 75 µg VP28 was mixed well with 1 mL complete Freund's adjuvant (Sigma-Aldrich, F5881) and subcutaneously injected into two rabbits. For booster immunizations, rabbits were injected with 1 mL PBS buffer containing 60 µg VP28 mixed with 1 mL incomplete Freund's adjuvant (Sigma-Aldrich, F5506) four times every 2 weeks later. Two weeks after the last booster dose, the antiserum was collected, mixed with 0.02% sodium azide, and stored at -80˚C.

Evaluation of VP28-immunized serum

The obtained serum was next validated for the ability to detect VP28 in Western Blot and ELISA.

For Western Blot, the total protein samples from *E. coli* BL21(DE3)/pQE-30 and BL21(DE3)/pQE30-*vp28,* the purified VP28 sample, the total protein sample from *E. coli* BL21(DE3)/pET28a-*omp38* cells expressing 6xHis-Omp38 protein, and the total protein sample from healthy and WSSV-infected shrimps were loaded into wells of 15% polyacrylamide gels. The protein samples from healthy and WSSV-infected shrimps were prepared and provided by Research Institute for Aquaculture No.2 (Ho Chi Minh city, Vietnam). Briefly, to prepare shrimp sample, shrimp heads were collected and stored in 70% ethanol. The rostrums were removed, and the remaining parts of shrimp heads were heated at 60˚C for 20 minutes to allow the evaporation of ethanol. The sample was then homogenized in 100 mM PBS buffer, pH 7.4 with the ratio of 1 g shrimp in 2 mL PBS buffer. The homogenized sample was centrifuged at 13,000 rpm, 4˚C for 5 minutes and the supernatant was used for Western Blot analysis. The obtained serum and a goat anti-rabbit IgG (H+L) secondary antibody conjugated HRP (Invitrogen, 65-6120) were used as the primary and secondary antibodies, respectively, at the dilution of 1:10,000. The signal was detected using ImageQuant Las 500 system.

For ELISA, 100 µL coating buffer (0.1 M Na_2CO_3, 0.1 M $NaHCO_3$, pH 9.4) containing purified VP28 at different concentrations was added into wells of a 96-well plate. The plate was incubated at 25˚C for 3 hours. After that, unbound proteins were removed from wells and 100 µL blocking buffer (0.1% tween 20, 100 mM $Na_2HPO_4.2H_2O$, 18 mM KH_2PO4, 1.37 M NaCl, 27 mM KCl, 1% bovine serum albumin) was added. The plate was further incubated for 1 hour. The blocking buffer was removed and 100 µL of

the solution containing obtained serum at the dilution of 1:10,000 to 1:2,560,000 was added into wells. The plate was incubated at 25˚C for 1 hour. Next, the solution was replaced by 100 µL of the solution containing goat anti-rabbit IgG (H+L) secondary antibody conjugated HRP (Invitrogen, 65-6120) at the dilution of 1:10,000, and the plate was incubated at 25˚C for 1 hour. Then, the secondary antibody solution was removed and 100 µL of 1-StepTM Ultra TMB-ELISA solution (ThermoFisher Scientific) was added. After 15 minutes of incubation at 25˚C, 100 µL of 2 N H_2SO_4 solution was added. Finally, the absorbance at 450 nm (OD_{450}) of each well was measured using a Multiskan Ascent microplate reader.

RESULTS

Verifying the expression of VP28 in *E. coli* BL21(DE3)/pQE30-*vp28*

In plasmid pQE30-*vp28*, *vp28* gene was placed under the control of *lac* operator, thus the expression of VP28 was induced by adding 0.5 mM IPTG in the culture medium. The total protein, aggregate and soluble fractions from cell lysates were prepared and checked for the presence of VP28 using SDS-PAGE and Western Blot analyses. We found that an intensive band around 28 kDa was present in the total protein sample from BL21(DE3)/pQE30-*vp28* (Fig. 1, well 6) but absent in all negative control samples (Fig. 1, wells 1-5). This band was detected by Western Blot analysis with anti-His tag antibody. Since the VP28 was fused with 6xHis-tag, these data clearly indicated that the observed intensive band was VP28 protein. In addition, we also found that VP28 was expressed in both soluble and insoluble forms (Fig. 1, wells 7-8). For the reason that protein expressed in soluble form is usually correctly folded and can be dissolved well in solution, thus probably leading to a better immunization, we used the soluble fraction for futher experiments.

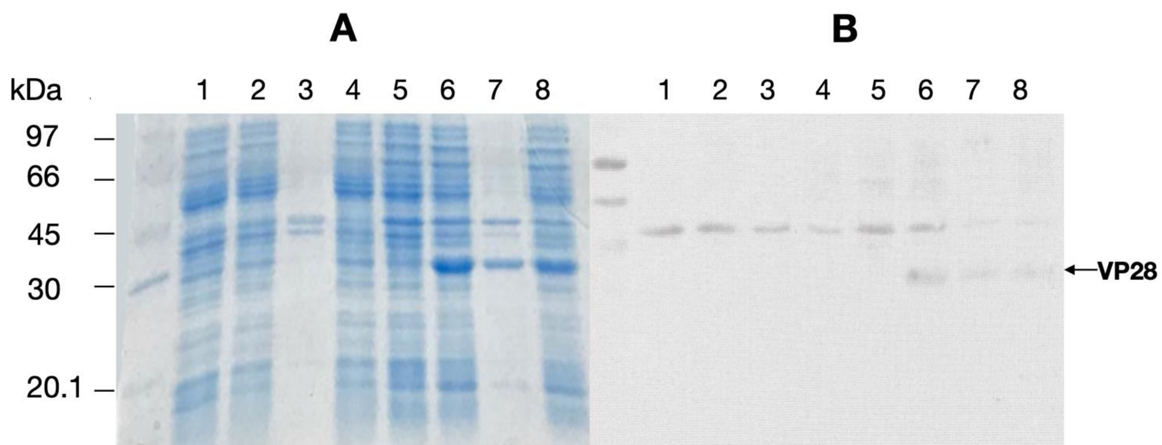

Figure 1. The expression of VP28 in E. coli was verified by SDS-PAGE (A) and Western Blot (B). 1: The total protein from IPTG-untreated BL21(DE3)/pQE-30; 2-4: The total protein, insoluble and soluble fractions respectively from BL21(DE3)/pQE-30; 5: The total protein from IPTG-induced BL21(DE3)/pQE30-*vp28*; 6-8: The total protein, insoluble and soluble fractions respectively from BL21(DE3)/pQE30-*vp28*.

VP28 purification

In order to prepare VP28 for rabbit immunization, the *E. coli* BL21(DE3)/pQE30-*vp28* strain was cultured in 300 mL LB-Amp medium and the expression of VP28 was induced by 0.5 mM IPTG. After that, the cells were collected and lysed by sonication. The supernatant fraction of cell lysate was obtained and used for VP28 purification with Ni-NTA chromatography. The purification result showed that VP28 was eluted at the concentrations of imidazole in the elution buffer of 250 mM and 500 mM (Fig. 2, wells 8-9). Due to the high purity of VP28 in 500 mM imidazole fraction (~85%), this fraction was used for the immunization in rabbits.

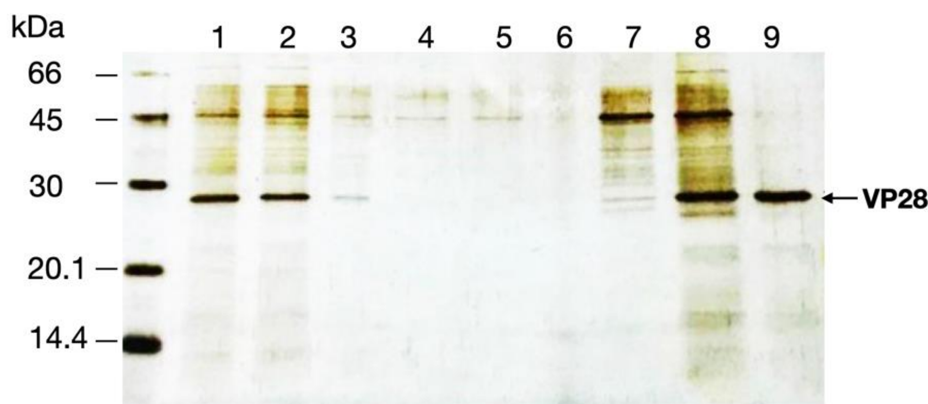

Figure 2. Analysis of protein fractions during VP28 purification. 1: Soluble fraction from *E. coli* cell lysate; 2: Flow-through fraction; 3: Wash fraction; 4-9: Elution fractions at 50 mM, 100 mM, 150 mM, 200 mM, 250 mM, and 500 mM imidazole, respectively.

Verifying the presence of VP28 specific antibodies in immunized serum

The purified VP28 was injected into rabbits to trigger their immune response for antibody production. The presence of VP28 specific antibodies in rabbit serum was verified by Western Blot, in which only one band around 28 kDa was detected in VP28 containing samples, including BL21(DE3)/pQE30-*vp28* and purified VP28 samples (Fig. 3, wells 4-5). This band was not observed in all negative control samples (Fig. 3, wells 1-3). In addition, in order to check whether the produced antibody can bind to 6xHis-tag, we used a protein sample from *E. coli* cells expressing the 6xHig-tagged outer membrane protein Omp38 (6xHis-Omp38) from *A. hydrophila,* which yielded no Western Blot signal, indicating that the obtain antiserum could recognize the recombinant VP28 but not the 6xHis fusion tag. Notably, the Western Blot analysis of WSSV-infected shrimps showed a clear band at the same size as the purified VP28 (Fig. 4). These data demonstrated that the obtained antiserum could recognize both the recombinant and native VP28.

Evaluation of antiserum affinity by ELISA

The affinity of antiserum to VP28 was evaluated by ELISA, in which VP28 at different amounts ranging from 0.39 ng to 200 ng were coated into wells of a microtiter plate and incubated with the obtained

antiserum at different dilutions. We found that the OD_{450} values of wells incubated with pre-immunized serum were all below the cut-off value (<0.15) whereas most of the wells incubated with immunized serum had a high OD_{450} value (up to 1.948 as in well coated with 200 ng VP28 and incubated with antiserum at the dilution of 1/10,000). This result indicated that the obtained antiserum can be used to detect VP28 in ELISA. Antiserum at the dilutions of 1/10,000 to 1/40,000 could recognize VP28 at all tested concentrations and antiserum at the dilution of 1/1,280,000 could still detect VP28 at high concentration (≥50 ng/well), but antiserum at the dilution of 1/2,560,000 could not recognize VP28 at any concentration. These data suggested that the sensitivity of obtained antiserum, or the lowest concentration of VP28 that can be detected by antiserum, was <0.39 ng/well; and its titer, or the lowest dilution of antiserum that can still be used to detect VP28, was 1/1,280,000.

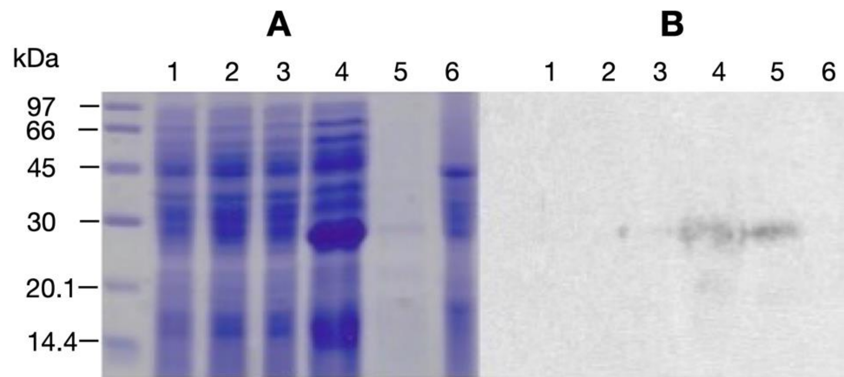

Figure 3. Evaluation of antiserum for the ability to recognize recombinant VP28 by SDS-PAGE (A) and Western blot (B). 1: The total protein sample from uninduced BL21(DE3)/pQE-30 cells; 2: The total protein sample from IPTG induced BL21(DE3)/pQE-30 cells; 3: The total protein sample from uninduced BL21(DE3)/pQE30-*vp28* cells; 4: The total protein sample from IPTG-induced BL21(DE3)/pQE30-*vp28* cells; 5: purified VP28; 6: The total protein from IPTG-induced *E. coli* BL21(DE3)/pET28a-*omp48*.

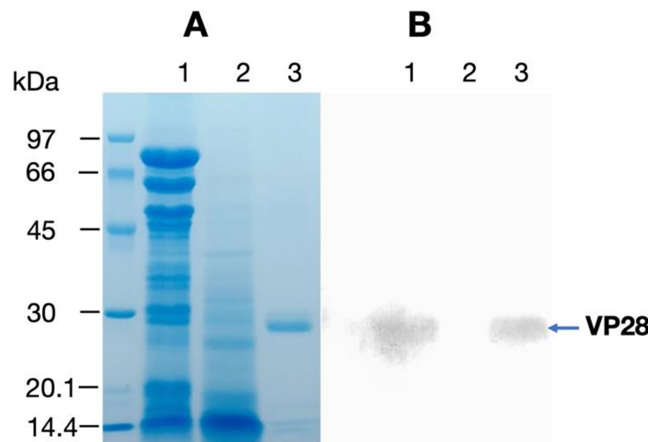

Figure 4. Evaluation of antiserum for the ability to recognize native VP28 in WSSV-infected shrimps by SDS-PAGE (A) and Western blot (B). 1: The total protein samples from WSSV-infected shrimps; 2: The total protein samples from healthy shrimps; 3: Purified recombinant VP28.

Table 1. OD_{450} values of wells containing different amounts of VP28 and incubated with the obtained antiserum at different concentrations (Data were present as mean±SD of three replicates).

Dilution fold of antiserum	The amount of Vp28 in well (ng/well)										
	200	100	50	25	12.5	6.25	3.13	1.56	0.78	0.39	0
(-)* 1/10,000	0.089±0.011	0.088±0.011	0.08±0.009	0.073±0.007	0.083±0.003	0.071±0.003	0.086±0.013	0.085±0.017	0.086±0.015	0.076±0.01	0.071±0.005
1/10,000	1.948±0.077	1.853±0.108	1.757±0.072	1.722±0.044	1.628±0.077	1.31±0.016	1.035±0.112	0.72±0.121	0.448±0.059	0.307±0.01	**0.108±0.025
1/20,000	1.726±0.114	1.652±0.057	1.569±0.079	1.512±0.137	1.235±0.042	1.106±0.045	0.85±0.103	0.543±0.063	0.348±0.05	0.227±0.015	0.082±0.023
1/40,000	1.404±0.148	1.38±0.081	1.247±0.056	1.153±0.058	0.981±0.048	0.82±0.076	0.567±0.056	0.393±0.051	0.249±0.031	0.172±0.018	0.071±0.02
1/80,000	1.117±0.101	1.149±0.12	0.945±0.115	0.872±0.086	0.747±0.085	0.558±0.089	0.426±0.099	0.273±0.054	0.184±0.04	0.134±0.014	0.063±0.008
1/160,000	0.844±0.079	0.772±0.091	0.695±0.038	0.566±0.075	0.47±0.081	0.354±0.068	0.278±0.056	0.186±0.05	0.136±0.032	0.114±0.025	0.059±0.004
1/320,000	0.480±0.093	0.417±0.099	0.35±0.094	0.292±0.104	0.232±0.075	0.171±0.071	0.13±0.047	0.099±0.036	0.078±0.017	0.079±0.022	0.059±0.002
1/640,000	0.298±0.017	0.256±0.032	0.223±0.048	0.159±0.028	0.136±0.028	0.103±0.025	0.086±0.015	0.073±0.006	0.067±0.016	0.066±0.005	0.057±0.002
1/1,280,000	0.224±0.043	0.172± 0.026	0.161± 0.033	0.113±0.015	0.104±0.016	0.078±0.014	0.078±0.011	0.067±0.007	0.06±0.009	0.066±0.006	0.06±0.005
1/2,560,000	0.131±0.005	0.117±0.008	0.104±0.006	0.078±0.008	0.085±0.008	0.078±0.013	0.074±0.009	0.071±0.008	0.062±0.007	0.057±0.002	0.055±0.005

* (-): Pre-immunized antiserum. Cut-off value=0.15, determined as mean± 3 SD of OD_{450} values from VP28-free samples incubated with antiserum at the dilution of 1/10,000 (**). The grey cells indicate OD_{450}>cut-off value.

DISCUSSION

This study was carried out in an attempt to produce a specific antiserum against WSSV envelope protein VP28. In order to prepare an adequate amount of antigen for immunization, we chose *E. coli* as the host strain for VP28 production due to its advantages such as fast growth, easy genetic manipulation, and high yield of recombinant protein expression. The SDS-PAGE and Western Blot results showed that we successfully established an *E. coli* strain capable of expressing recombinant VP28 (Fig. 1). However, besides the signal of target protein, we also saw another band around 45 kDa in all lanes on the Western blot membrane. We reasoned that since the primary antibody used in this experiment is specific to histidine, this antibody can also bind to Histidine-rich proteins, which are present at a high degree in *E. coli* cells (Robichon *et al.*, 2011). In addition, we also found that VP28 was expressed as both soluble and insoluble forms in *E. coli*. Some previous studies demonstrated that antigens in aggregate form could trigger immune response with greater efficiency, but antibodies produced against folded antigens could react best with antigens in their native forms. Therefore, with the aim of producing an antiserum for the detection of native VP28, we chose the supernatant fraction of *E. coli* cell lysate to obtain soluble VP28.

Since VP28 was fused with 6xHis-tag, the VP28 protein was purified using the Ni-NTA affinity chromatography. After purification, VP28 was obtained with high purity (~85%). The purity of antigen is an important feature to determine the specificity of produced antibodies. According to the guidelines of Davids Biotechnologie GmbH, an antibody-producing company, protein with the purity >70% could be used for immunization of animals for antibody production (DavidsBiotechnologie). Thus, the purity of

obtained VP28 (~85%) was acceptable for producing anti-VP28 antiserum in the next step.

In this study, rabbits were chosen as the host species for antibody production since they are easy to handle and able to provide a large quantity of antisera with high-titer and high-affinity antibodies at a reasonable cost. Rabbits were immunized with VP28 following a 90-day schedule, including one initial shot and 4 booster shots. The Western Blot demonstrated that the obtained antiserum could detect both the recombinant VP28 and the native VP28 from WSSV-infected shrimps. The antiserum could also recognize VP28 in ELISA with the sensitivity of <0.39 ng/wells VP28 and the titer of 1/1,280,000. Importantly, we had established a yeast strain expressing VP28 on its cell surface as reported in our previous study (Linh *et al.*, 2021), in which this antiserum was also used to verify the presence of VP28 on the yeast cell surface in an immunofluorescence staining assay, suggesting the application of the obtained antiserum in this method.

Several previous studies also reported the production of rabbit polyclonal antibody against VP28. Van Hulten *et al.* (2001) demonstrated that the obtained polyclonal antibody reacted with VP28 but not the other proteins of WSSV nucleocapsid. Yoganandhan *et al.* (2004) showed that the polyclonal antiserum could be used to detect VP28 in all organs of WSSV-infected shrimps. However, in these studies, the reactivity of produced antibody was only verified by Western Blot analysis. We here demonstrated that the obtained antiserum can be applied to detect VP28 in Western Blot, ELISA, and immunofluorescence staining.

CONCLUSION

In this study, we successfully produced an antiserum from rabbits that can recognize VP28 in Western Blot and ELISA (sensitivity of <0.39 ng/wells VP28, titer of 1/1,280,000), which can facilitate future studies on this potential antigen. Besides, this antiserum can also be further evaluated for the application in developing an effective kit to detect WSSV in shrimp samples.

Conflict of interest: *The authors declare that they have no conflict of interest.*

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