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Design of Tandem Epitope Vaccine Against White Spot Syndrome Virus

Huaiyu Chen[1, 2], Le Dong[1, 2], MengTing Wang[3], Yongzhong Lu[3,*], Fang Wang[1, 2,*]

[1]Fujian Province Key Laboratory for the Development of Bioactive Material from Marine algae, Quanzhou Normal University, Quanzhou, China, 362000
[2]College of Oceanology and Food Science, Quanzhou Normal University, Quanzhou, 362000, China
[3]Qingdao University of Science and Technology, Biology Department, Qingdao, China, 266042

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Corresponding Authors
Tel.: +8618661908889
+8613159075530
E-mail: luyz@qust.edu.cn
dwf320@163.com

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Abstract

White spot syndrome virus (WSSV) has long been a major threat on shrimp industry, but there is always a lack of effective prevention and control methods. Vaccines are widely regarded as the safest and most effective methods for preventing WSSV, and some main envelope proteins have showed this potential. In order to combine the antigenicity of these proteins together for better vaccine performance, in this study a strategy commonly used in vertebrates was tried in shrimp. The dominant epitopes of five envelope proteins VP19, VP24, VP26, VP28 and VP41A were predicted and combined for tandem epitope vaccine design by bioinformatics methods, the designed vaccine gene was chemically synthesized and expressed in *Escherichia coli*, then the recombinant peptide was purified for function evaluation in shrimp. The results showed that the designed peptide had good flexibility, hydrophily and antigenicity with all the epitope regions accessible; the synthesized gene was linked with the tag sequence and successfully expressed in BL21(DE3), and the purified fusion peptide showed a positive effect in WSSV prevention. This research provided a promising method to design vaccines against WSSV, and further work in genetic engineering should be done so as to make it operable for application in shrimp farm.

Introduction

White spot syndrome virus (WSSV) is one of the most virulent pathogens of shrimp, which once led to serious economic losses in aquaculture industry (Domínguez-Borbor et al., 2019). Currently there is still no effective method to prevent, treat, or control WSSV infection (Feng et al, 2018). As known in vertebrates, development of vaccine is the most effective way to prevent virus infection. Though lack of acquired immunity, there is increasing evidence that specific immune response called 'innate immunity with specificity' or 'immune priming' exists in shrimp and other arthropods, and exhibits somewhat immune specificity and immune memory (Ng et al. 2015). Many efforts have been made to develop vaccines for WSSV prevention, besides inactivated whole virus vaccine, most researches have focused on subunit vaccines development (Feng et al, 2017).

There are about 30 envelope proteins in WSSV, which play an important role in virus infection as well as in triggering host defenses (Chang et al., 2010; Ma et al., 2019; Yang et al. 2012), and have been the main targets for vaccines development. To date, some main envelope proteins have been prepared as recombinant subunit vaccines (Ananphongmanee et al. 2015; Ha et al. 2008;

Solís-Lucero et al. 2016; Thomas et al. 2014; Yang et al.2012), as well as DNA or RNA-based vaccines (Ahanger et al. 2014; Ning et al. 2009). Among which, the major envelope proteins VP26 and VP28 have drawn the most attention and provided significant protection against WSSV (Feng et al, 2018; Ma et al. 2019; Shine et al, 2020; Solís-Lucero et al. 2016); additionally, VP19 and VP24 also have showed the potential in WSSV prevention (Lu et al.2008; Thomas et al. 2014). Moreover, some studies indicated combined subunit vaccines showed better performance than single ones, and should be given more attention (Lei et al. 2021; Weerachatyanukul et al. 2021). However, till now, no subunit vaccines have claimed more than 50% of protection efficiency. Epitope screening as well as epitope-based vaccine design provides a novel approach for developing combined vaccines against WSSV by targeting the envelope proteins (Momtaz et al.2019; Shine et al, 2020).

In this study, for the purpose of developing a new weapon against WSSV, the vaccine was designed using a strategy commonly used in vertebrates, the dominant epitopes of envelope proteins VP19, VP24, VP26, VP28 and VP41A (Xie et al.2015) were screened by bioinformatic methods, and tandemly expressed in *Escherichia coli* for vaccine function evaluation. Successful design of a peptide vaccine will provide the candidate for oral vaccine development through genetic engineering so as to improve its practical application.

Materials and Methods

Materials

The pSmart-I plasmid (General Biosystems, USA) was used as an expression vector, which carried 6× His-tag and SUMO to facilitate solution and purification of the recombinant expressed proteins (Yu et al. 2017). *E. coli* BL21 (DE3) was used as the host for DNA manipulation and expression of foreign genes.

Linear epitope prediction

The protein sequences of VP19, VP24, VP26, VP28 and VP41A were retrieved from the NCBI database (YP_009220610.1, YP_009220476.1, YP_009220584.1, YP_009220614.1, YP_009220554.1). ABCpred (Saha et al. 2006) and BepiPred-2.0 (Jespersen et al. 2017) servers were employed for epitope prediction, with the threshold value being set 0.85 and the length of the linear epitopes being set from12 to 22. The epitopes predicted by both servers were selected for novel vaccine design.

The epitopes selected from the five envelope proteins were linked virtually with the flexible peptide fragment “GGGG”, and the protean program in DNAstar was used to evaluate the flexibility, hydrophily and surface probability of different epitope combinations (Burland, 2000). The 3D structure of the designed vaccine was built via Swiss-model server (Waterhouse et al. 2018).

Construction of Expression Vectors

The amino acid sequence of the designed peptide was converted to DNA sequence, and the codons were optimized to the bias of *E. coli* through the server ExpOptimizer (https://www.novopro.cn/tools/codon-optimization.html). This DNA sequence was synthesized by Synbio Technologies (Suzhou, China) to be used as a template for PCR amplification. Two primers were designed to cover both ends of the synthesized DNA sequence, with BamHI site added to the 5’ end of forward primer, and XhoI site added to the 5’ end of reverse primer (F:CG*GGATCC*CAACTGACCTACGCGG TCAAG; R:CC*CTCGAG*GGTTAGCCAATGCCGCTTTTGG). PCR was performed using Taq DNA polymerase (Takara) under conditions: 95°C for 3 min, 95°C for 30 s, 65°C for 30 s, 72°C for 30 s of 30 cycles. After digestion with BamHI and XhoI at 37°C, the purified PCR products were linked with the linearized pSmart-I plasmid between the BamHI-XhoI site to fuse with N-terminal SUMO tag.

Bacterial Expression of SUMO-Vaccine Fusion Peptide

The plasmid pSmart-I-vaccine was transformed into competent *E. coli* BL21(DE3) by heat shock method, the transformant was screened on ampicillin LB plate and confirmed by PCR, the positive one was inoculated and cultivated at 37°C in LB liquid medium containing 50 μg/mL ampicillin in subsequent experiments. The culture was transferred into fresh LB medium at a 1:100 ratio and cultivated with shaking at 220 rpm until OD_{600} reached 0.6–0.8. In order to find the optimal concentration, different amount of isopropyl β-d-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich) was added to the cultures to induce the expression of the fusion peptide for 5 h.

Samples were boiled for 30 min with loading buffer and the expression of the fusion peptide was detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE).

Purification of the Fusion Peptide

The cells were harvested by centrifugation (12000 rpm; 4°C; 5 min), and lysed by sonication (100W, 50 cycles; 10 s per cycle; 1 min interval) in cold 25 mM Tris buffer (300 mM NaCl, pH 8.0); after centrifugation (12000 rpm; 4°C; 15 min), the supernatant was loaded onto a 10 mL Ni-IDA -Sepharose CL-6B affinity column (Clontech), elution was conducted by a step gradient of imidazole from 10 to 400 mM at 2 mL/min. The collected peptide solution was stored at −20°C before SDS–PAGE and Western Blot analyses

Western Blot Analysis

Western Blot was performed to confirm the fusion peptide. Briefly, proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane, after being

immersed in TBS (150 mM NaCl, 50 mM Tris-HCl) containing 0.05% Tween 20 and 5% milk at 4°C overnight, the membrane was incubated with anti-his primary antibody solution (1:1000) and the followed goat anti-mouse secondary antibody solution (1:5000). After a washing, the membrane was developed using ECL detection system.

Detection of WSSV by Conventional PCR

Total DNA was extracted from the frozen gill tissue of each shrimp using the genomic DNA purification kit (Sangon Biotech, Shanghai, China), and stored at -20℃ until use. WSSV specific primers (WF: GTAGTGGTTGT TGGTGGATG; WR: CCTGGTGCTTGTGAAATGTG) were used to amplify WSSV with the expected size of 1kb. The conditions used for amplification were as follows: 95°C for 3 min, 95°C for 30 s, 58°C for 30 s, 72°C for 60 s of 30 cycles. Known negative and positive controls were included in all the reactions.

Vaccination with the Recombinant Peptide

WSSV-free *Litopenaeus vannamei* (average body weight 10 g) were purchased from the shrimp farm, Rizhao, Shandong, China. They were kept in tanks at 20±0.5℃, and supplied with clean seawater as well as commercial feed per day. After 10 days, they were divided into three groups (50 in each group), including blank control, negative control and the experimental group, and three replicates were set. Each in the experimental groups was injected with 100 μL peptide solution (1mg/mL), while those in the negative control groups was injected with the same volume of peptide buffer. On the 5th day postvaccination, both the negative control and the experimental groups were fed with tissues of WSSV-infected shrimp (~10^6 copies/g) (Lu et al. 2020). 10 days later the protection efficiency was calculated as follows: (incidence rate of control groups - incidence rate of vaccination groups)/incidence rate of control groups * 100%, natural death in the blank control groups could be used to correct the incidence rate.

Statistical Analysis

Difference analysis between the negative control and the experimental groups was made by T-test, and the statistical significance was declared at $P<0.05$.

Results

Linear Epitope Prediction

Nearly 400 epitope fragments with score over 0.85 were predicted from the five envelope proteins. The positions of most epitopes predicted by the two servers were not exactly the same, only those predicted by the two servers with the highest scores were considered for vaccine design. Finally, two fragments from VP19 (61-74, 80-91), two from VP24 (31-42, 82-95), two from VP26 (62-75,98-111), two from VP28 (82-93, 152-162), and four from VP41A (154-167, 213-226, 236-247, 247-258) were remained.

In total, 12 epitopes from five envelope proteins were combined in a single peptide by the linker "GGGG" fragment randomly, and the resultant sequence with good flexibility, hydrophily, surface probability, and antigenicity was used for vaccine development (Figure 1a,b) ; The 3D structure of this sequence built via Swiss-model server also showed it was in a relaxed state with all the epitope regions accessible (Figure 1b).

Expression of the Designed Vaccine Peptide

Based on the peptide sequence, the gene of the designed vaccine was chemically synthesized and expressed in *E. coli* for function testing (Additional file 1). Initially, an expression vector was constructed by inserting the vaccine gene between NdeI and XhoI of pET-28a (+), no obvious expression was detected in the host BL21(DE3) or Arctic Expression (DE3) by SDS-PAGE analysis (data not shown), then a vector for expressing N-terminal SUMO–tagged vaccine peptide was constructed, with the expression to be driven under the control of the IPTG-inducible T7 promoter. The fusion peptide was successfully expressed in BL21(DE3), which was confirmed by Western Blot (Figure 2a, b). IPTG from 0.05 to 0.3 mM all could induce the expression, and 0.2 mM IPTG was used for fusion peptide preparation. The vaccine peptide was obtained with his-tag affinity purification, and the purified peptide had the purity over 90% as estimated by SDS-PAGE (Figure 3).

Influence of the Vaccine Peptide on WSSV Challenged Shrimp

4-5 days after WSSV challenging, shrimps in the negative control groups displayed signs of reduction in food consumption, vigor, and red discoloration of body. About 10 days later, there were several natural deaths in the blank control groups, 22±2 survivors in the negative control and significantly more (32±2) survivors in the experimental groups ($P<0.01$). PCR analysis showed that WSSV could be detected from the dead shrimps of the negative groups and the experimental groups, whereas it couldn't be detected from the blank groups and the survivors of the experimental groups. These results indicated that the peptide could provide a protection against WSSV, the calibrated protection efficiency could reach to 35.7% (Figure 4 a, b), higher than that of VP28 reported by Zhang JY, et al (2012).

Discussion

In recent years, reverse vaccinology combining immunogenomics and bioinformatics is widely used in developing novel vaccines due to high efficiency and low

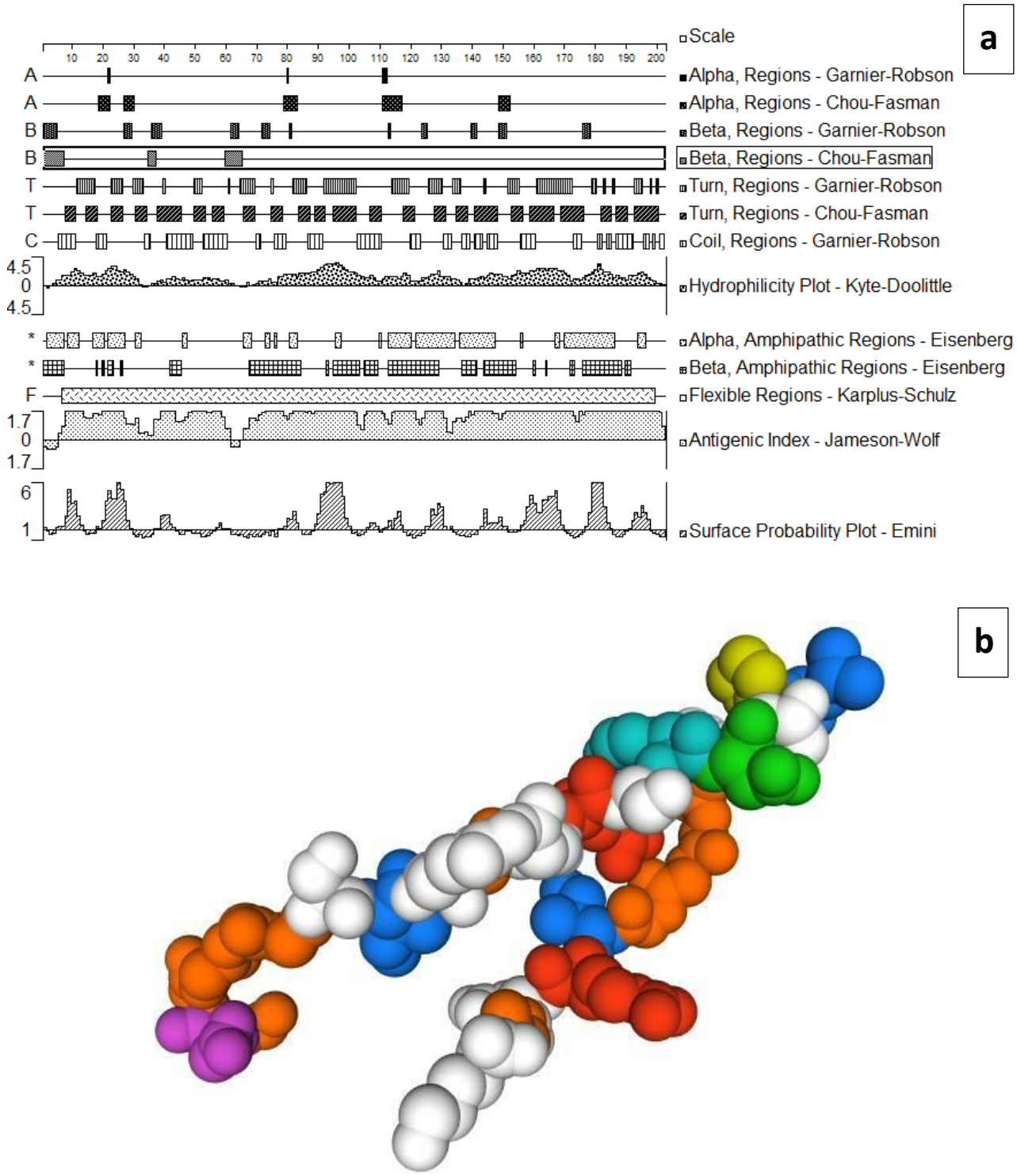

Figure 1. Bioinformatic analysis of the designed vaccine

a. Immunogenicity evalutation of the designed vaccine by protean software;
The designed peptide was predicted to be composed of alpha regions, beta regions, turn regions and coil regions, and have good hydrophilicity, flexibility, surface probability and antigenicity according to each index.

b. 3D structure analysis of the designed vaccine by Swiss-model server
The structure was displayed in spacefill style and Clustal color scheme, and it showed that the designed peptide was in a relaxed state with all the epitopes accessible.
The amino acids sequences of the designed vaccine:
qltyavknnneytegggg**nkkldkkdkday**gggg**tvtaprtdpagtgaen**gggg**ssntssftpvs**gggg**kvdcetgdieeeyn**gggg**ndnddedkyknr**gggg**qgtsdselvkkkgd**ggg**g**sirgersyntplgk**gggg**irngksdaqmke**gggg**kkdsdsdtdkdtdd**gggg**ireydkpknef**gggg**esdsdttksgig**
The bold regions linked by "gggg" were epitopes from VP41A, VP24, VP26, VP28, VP41A, VP19, VP24, VP26, VP28, VP19, VP41A and VP41A in order.

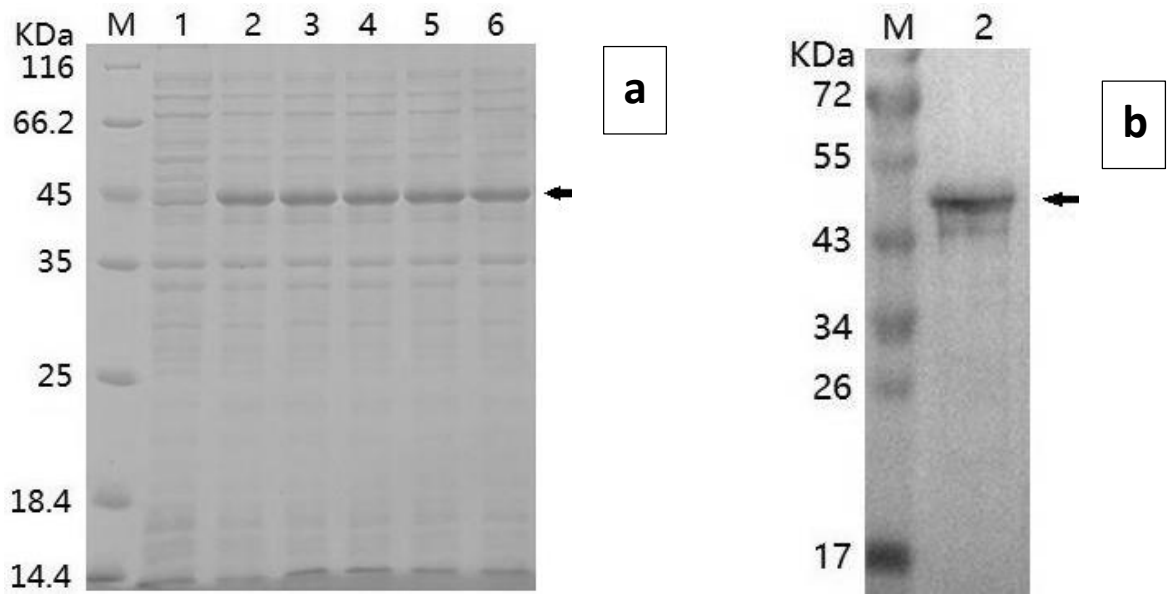

Figure 2. Fusion expression of the designed vaccine in *E. coli*

a. The expression of the fusion peptide was induced by IPTG
M: protein marker; 1-6: 0, 0.05, 0.1, 0.15, 0.20, 0.5 mM IPTG

b. The fusion expression was confirmed by Western Blot with his tag antibody
The arrow indicted the target product.

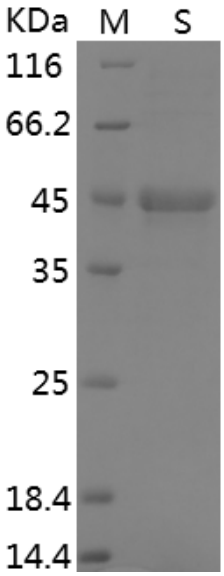

Figure 3. SDS PAGE analysis of the purified fusion peptide

The purity of the purified peptide was over 90% estimated by SDS-PAGE
M: protein marker; S: the purified fusion peptide with the molecular weight of 45KDa

cost (Sanami et al. 2020). The next-generation epitope-based vaccines are making progress not only in severe human disease prevention, but also in other fields such as aquaculture and stock farming (Baliga et al. 2018; Kar et al. 2018; Momtaz et al.2019). Epitopes are short amino acid sequences of a protein that can induce a more direct and potent immune response. In vertebrates, T and B-cell epitopes of antigen proteins could be predicted and screened based on the antigenicity, toxicity, allergenicity, and cross-reactivity with human proteomes using immunoinformatic analysis. Epitope-based vaccines can be designed by combining epitopes from different antigens in a single peptide by the appropriate linker, which will be potential in providing cross protection, as well as resistance to the ever-evolving viruses (Parvizpour et al. 2020).

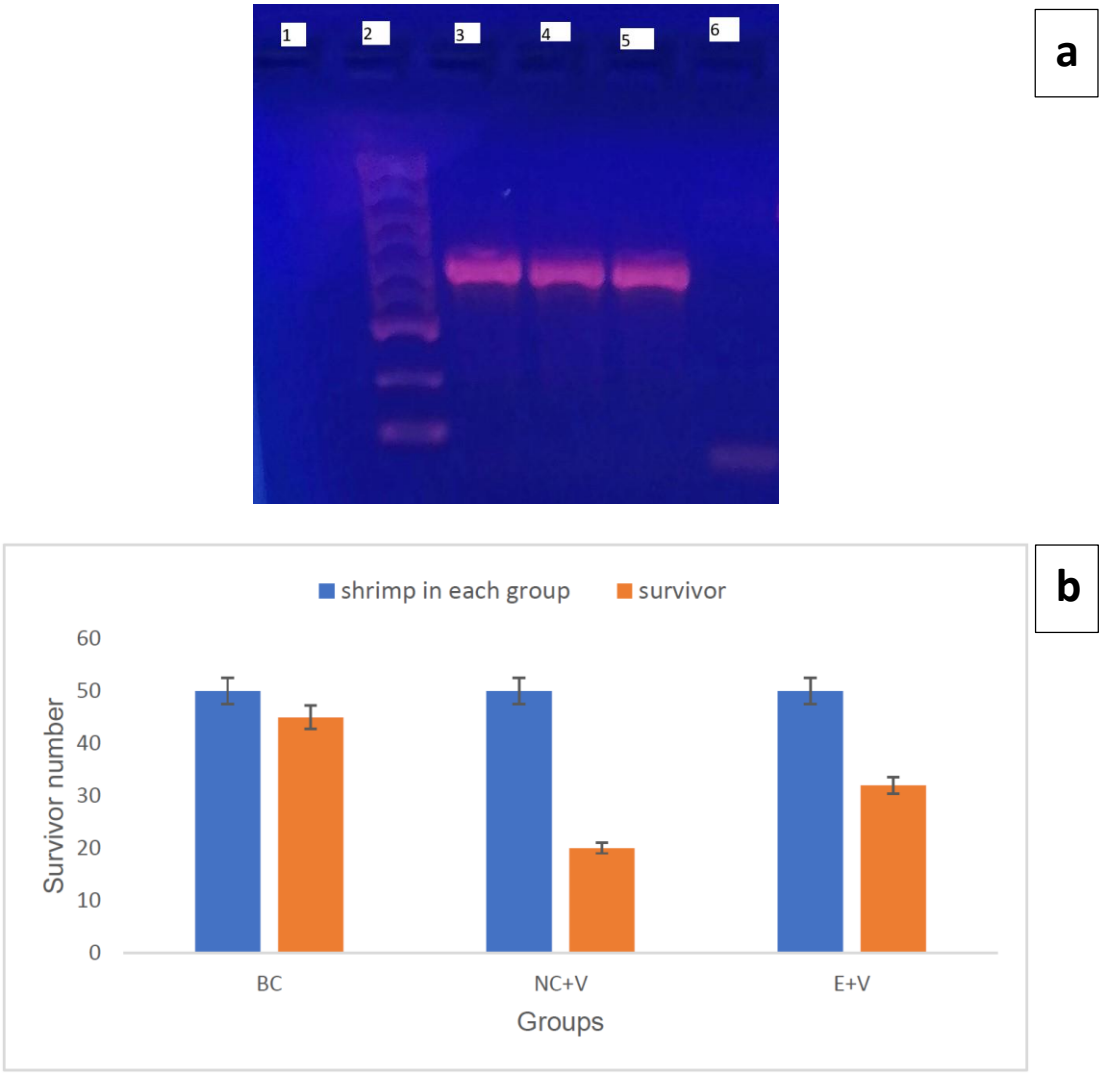

Figure 4. Evaluation on the effect of the designed vaccine

a: detection of WSSV from different groups by PCR

1 blank; 3 positive control; 4 dead shrimp from the negative group; 5 dead shrimp from the experimental group; 6 a survivor from the experimental group;

2 DNA marker (100, 250, 500, 750, 1000, 1500, 2000, 3000, 5000 bp)

b: influence of the peptide on the survival of WSSV-challenged shrimp

B C: blank control; NC+V: blank control group; E+V: experimental group
10 days after peptide injection, difference between the negative control (n=3) and the experimental groups (n=3) was significant (P<0.01, T-test)

To our knowledge, epitope-based strategy has not been applied in shrimp vaccine design, which might be mainly due to the worry about the immunity difference between vertebrates and shrimp. Increasing evidence indicated that the innate immunity in shrimp exhibited somewhat immune specificity, and a hypervariable protein Down syndrome cell adhesion molecule (Dscam) has been identified as a pathogen-specific recognizing molecule (Ng et al. 2015). The efficiency of the killed vaccines and subunit vaccines further encouraged us to develop a more effective epitope-based vaccine following the strategy in vertebrates. Epitope identification is the first step for this strategy, but it is limited to the availability of monoclonal antibodies (Shine et al, 2020). Momtaz et al. (2019) recently initiated design of epitope-based vaccine against WSSV by an immunoinformatic approach, and this pioneering work will encourage more exploration in this field.

Based on this strategy, a novel vaccine can be designed by combining epitopes from different antigens in a single peptide. VP19, VP24, VP26 and VP28 have been expressed to act as subunit vaccines and showed protection effects against WSSV (Ananphongmanee et al. 2015; Ha et al. 2008; Ma et al. 2019; Solís-Lucero et

al. 2016; Thomas et al. 2014; Yang et al.2012). A vaccine possessing epitopes from them may be more potential in WSSV prevention. VP41A is also an important envelope protein mediating WSSV infection (Lee et al. 2017), though no report about its vaccine function is available now, it was also considered in this study.

Multiple epitopes were predicted in each envelope protein, if all were remained in a single peptide, some epitopes might be disguised. Generally, the outside region of an envelope protein is considered to be suitable for vaccine design, TMHMM server analysis showed VP28 was composed of a short outside region (4 amino acid), a transmembrane region (23 amino acid) and an inside region (177 amino acid), but the studies have showed VP28 could provide a significant protection against WSSV, which suggested when screening the epitopes from the envelope proteins of WSSV, the outside region should not be the sole target. When mediating the viral entry, the envelope proteins usually underwent conformational changes, which might lead to the exposure of inside regions. Du et al. (2013) reported the middle region (residues 35-95) was essential to maintain the neutralizing linear epitopes of VP28 and responsible in eliciting immune response, so the epitopes from middle region and inside region of VP28 (82-93, 152-162) were selected.

In this study preliminary results indicated that the designed peptide showed a protective effect on shrimp after feeding with WSSV-infected shrimp tissues, indicating the feasibility of our vaccine design strategy. In order to further improve the protection efficiency, the peptide should be optimized by effective epitope selection and combinations. With the increasing of our knowledge on shrimp immunity, more accurate epitopes will be identified, and more effective vaccines will be designed. Furthermore, genetic engineering will make it feasible to apply in shrimp farm as an oral vaccine.

Conclusions

Above all, an epitope-based vaccine design strategy was applied in shrimp for the first time, and the positive effect of the designed peptide in WSSV prevention indicated this new avenue is promising in shrimp vaccine development.

Ethical Statement

This article does not contain any studies with animals performed by any of the authors.

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Author Contribution

Huaiyu Chen and Le Dong contributed equally to this work on material preparation and experiment, Mengting Wang contributed to bioinformatic analysis, Yongzhong Lu contributed to the study conception and design, Fang Wang contributed to project administration.

Conflict of Interest

The authors declare that they have no conflict of interest.

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