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Identification of three novel B-cell epitopes of VMH protein from *Vibrio mimicus* by screening a phage display peptide library

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ABSTRACT

Vibrio mimicus is the causative agent of ascites disease in fish. The heat-labile hemolytic toxin designated VMH is an immunoprotective antigen of *V. mimicus*. However, its epitopes have not been well characterized. Here, a commercially available phage displayed 12-mer peptide library was used to screen epitopes of VMH protein using polyclonal rabbit anti-rVMH protein antibodies, and then five positive phage clones were identified by sandwich and competitive ELISA. Sequences analysis showed that the motif of DPTLL displayed on phage clone 15 and the consensus motif of SLDDDST displayed on the clone 4/11 corresponded to the residues 134–138 and 238–244 of VMH protein, respectively, and the synthetic motif peptides could also be recognized by anti-rVMH-HD antibody in peptide-ELISA. Thus, both motifs DPTLL and SLDDDST were identified as minimal linear B-cell epitopes of VMH protein. Although no similarity was found between VMH protein and the consensus motif of ADGLVPR displayed on the clone 2/6, the synthetic peptide ADGLVPR could absorb anti-rVMH-HD antibody and inhibit the antibody binding to rVMH protein in enhanced chemoluminescence Western blotting, whereas irrelevant control peptide did not affect the antibody binding with rVMH. These results revealed that the peptide ADGLVPR was a mimotope of VMH protein. Taken together, three novel B-cell epitopes of VMH protein were identified, which provide a foundation for developing epitope-based vaccine against *V. mimicus* infection in fish.

1. Introduction

Vibrio mimicus (V. mimicus) is an intestinal pathogen that not only cause ascites disease in fish (Geng et al., 2014; Zhang et al., 2014), but also induce sporadic acute gastroenteritis and food poisoning in humans after the consumption of raw or undercooked aquatic products (Chitov et al., 2009). The ascites disease in fish is very common, which results in serious losses to the aquaculture industry and threatens public health. Thus, the development of a novel vaccine, such as epitope-driven vaccine prepared with multiple epitopes of protective antigen from the pathogen, to prevent ascites disease in fish is vitally important for the aquaculture industry.

Although *V. mimicus* is known to produce a variety of extracellular toxic factors, the most common factor is a heat-labile hemolytic toxin designated VMH which is immunologically similar to El Tor hemolysin (Honda et al., 1987). Miyoshi et al. (1997) firstly purified the VMH protein from the culture filtrate of *V. mimicus* and documented that VMH simultaneously possesses hemolytic and enterotoxic activity. Subsequent studies indicated that the *vmh* gene is present in all strains isolated from various clinical and environmental specimens (Bi et al., 2001; Iliana et al., 2014). VMH protein is a relative conserved protective antigen which can be served as vaccine candidate (Li and Wu, 2007). In our previous studies (Wang et al., 2010), full-length *vmh* gene was cloned, sequenced and analyzed. It was found that *vmh* complete sequence contains a 2235 bp open reading frame, encoding VMH protein of 745 amino acids. Moreover, N terminal 185–552 amino acid region of the protein is its hemolytic domain, but the epitopes of VMH protein are still unclear.

Knowledge of the locations of VMH protein epitopes is important for both the understanding of immunological events and the development of epitope-based vaccines, as well as diagnostic and therapeutic tools for *V. mimicus* infection. At present, phage display technology has been widely used in B-cell epitope screening (Rowley et al., 2004). With this approach, peptide is expressed as a fusion entity with a coat protein of bacteriophages, resulting in display of the fusion polypeptide on the surface of the virion, while

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the DNA encoding the fusion polypeptide within the virion. Phages displaying peptides are then allowed to interact with antibodies immobilized on a solid support, and the binding phages are then eluted and can be specifically enriched by several cycles of affinity selection. The identity of the fusion peptide can then be determined by sequencing the inserts present in the genome of the recombinant phage.

The aim of our study was to screen and identify the novel B-cell epitopes of VMH protein by scanning a commercially available phage displayed 12-mer peptide library with polyclonal anti-rVMH-HD antibody. Our findings may provide a basis to develop epitope-based vaccine against *V. mimicus* infection.

2. Materials and methods

2.1. Ethics statements

Animal experiment was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the national laboratory animal welfare ethics, and protocols concerning animals were approved by the Ethical Committee of the Faculty of Veterinary Science of Anhui Agricultural University (Permit Number: 20130402). Every effort was made to reduce the number of animals used and minimize the suffering of the animals.

2.2. Bacterial strains, plasmids and the phage peptide library

The *V. mimicus* 04-14 isolate was obtained from diseased *Ctenopharyngodon idella* in our previous study (Liu et al., 2015). The pET-32a(+) vector was purchased from Novagen, USA. The Ph.D.-12™ Phage Display Peptide Library Kit (Complexity $\sim 2.7 \times 10^9$ transformants, titre 1.5×10^{13} PFU/mL) containing *Escherichia coli* (*E.coli*) host strain ER2738 and ‒96 gIII sequencing primer was purchased from New England BioLabs. 04-14 isolate was cultured in brain heart infusion broth (BHI; Beijing Solarbio Science & Technology Co., Ltd., China) at 30 °C, while *E.coli* Rosetta (DE3) and ER2738 were cultured in Luria Bertani medium (Beijing Solarbio Science & Technology Co., Ltd., China) at 37 °C.

2.3. Expression, purification, renaturation and identification of VMH-HD fusion protein

Gene fragment *vmh* (1–1525 bp) containing hemolytic domain of VMH protein was amplified from the genomic DNA of *V. mimicus* 04-14 isolate by PCR using the primers P1 (5′CCG**GAATTC**ATGCCAAAACTCAATCGTTG3′, ***EcoR*I site underlined**) and P2 (5′CCG**CTCGAG**TTAGCGACCTTGATCATCAG 3′, ***Xho*I site underlined**). Then the fragments were cloned into the *EcoR* I and *Xho* I sites of pET-32a(+) vector. The recombinant plasmid pET-32a-*vmh* was verified by restriction enzyme digestion and sequencing, and then transformed into *E.coli* Rosetta (DE3) competence cells. The expression of the recombinant VMH-HD fusion protein was induced with 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma) at 16 °C. After 18 h induction, the cells were harvested by centrifugation at $4000 \times g$ for 10 min. The cell pellet was washed twice with ice-cold lysis buffer (20 mM of Tris-HCl, 500 mM of NaCl, and 5 mM of EDTA, pH 8.0) and resuspended in 3 mL of this buffer. Cell preparations were then sonicated (20×3 s with 3 s on ice between each cycle) at maximum amplitude. Insoluble and soluble fractions were separated by centrifugation at $10{,}000 \times g$ for 10 min at 4 °C. 15% SDS-PAGE was performed to monitor the expression of the recombinant VMH-HD fusion protein.

Recombinant VMH-HD fusion protein was expressed in the form of inclusion bodies. Inclusion bodies were collected by centrifugation at $10{,}000 \times g$ for 30 min and then washed twice with buffer A (20 mM Tris-HCl, 1 mM EDTA, 1% (v/v) Triton X-100, pH 8.0). The washed inclusion bodies were solubilized with buffer B, which was 25 mM Tris–HCl, pH 8.0, containing 8 M urea, and then were purified using a high-affinity Ni-NTA resin column (GE Healthcare Life Sciences) according to the manufacturer's instructions. For the renaturation of the denatured VMH-HD, the denatured protein solution was dialyzed against renaturation buffer (50 mM Tris-HCl, 2 mM EDTA, 0.5 M L-Arg, pH 8.0) at 4 °C and stirred for 48 h. The buffer was changed at least six times during renaturation.

After refolding, the hemolytic activity of rVMH-HD fusion protein was detected by hemolytic testing on plates as described previously (Gao et al., 2000). Briefly, TSA-blood agar plate, consisting of tryptone soya agar (Oxoid) supplemented with 7.5% (v/v) defibrinated *Ctenopharyngodon idella* blood, was prepared. The agar plate was plugged with oxford cup to form a hole of 8 mm in diameter, then 50 μL of rVMH-HD fusion protein (0.5 mg/mL) were added to hole, and incubated for 18–24 h at 30 °C. The hemolysis circle on fish blood agar plate was determined by direct visual observation. In addition, Western blotting was performed to evaluate the antigenicity of rVMH-HD fusion protein. After being separated by 12% SDS-PAGE, the purified protein was transferred onto a polyvinylidenefluoride (PVDF) membrane. Then, the membrane was blocked with 5% nonfat dried milk in PBS for 2 h at 37 °C, followed by incubation with purified rabbit anti-exotoxin of *V. mimicus* antibody at a dilution of 1:500 (The antibody was prepared in our previous studies according to the protocol provided by Miyoshi et al. (1997)) for 1 h at 37 °C. After three washes with PBST (PBS containing 0.05% Tween-20), the membrane was incubated with 1:4000 HRP-conjugated goat anti-rabbit IgG (Novagen) for 1 h at 37 °C. Color was developed with 3,3′-diaminobenzidine tetrahydrochloride (DAB) and the reaction was stopped by rinsing with distilled water and drying the membrane.

2.4. Preparation and purification of polyclonal anti-rVMH-HD antibodies

To prepare polyclonal antibody against rVMH-HD fusion protein, New Zealand white rabbit (2 kg) was subcutaneously immunized with 1 mg purified rVMH-HD fusion protein emulsified with an equal volume of Freund's complete adjuvant (Sigma). Booster injection containing purified rVMH-HD protein with equal volume of Freund's incomplete adjuvant (Sigma) were given at 2-week intervals. The final immunization, purified rVMH-HD fusion protein without adjuvant was given intraperitoneally. Serum were collected prior to immunization and on day 28 post immunization, respectively, then were purified by the combination of saturated ammonium sulfate precipitation and HiTrap Protein G HP Column (Pharmacia, Sweden) according to the manufacturer's instructions. The purity of the purified antibody was detected by 12% SDS-PAGE.

The purified antibody titer was determined by indirect ELISA. Briefly, 96-well plates (Corning, USA) were coated with 20 μg/mL purified rVMH-HD fusion protein, the purified antibody were diluted from 1:100 to $1{:}100 \times 2^{31}$ and added to wells in triplicate, and negative control (unimmunized normal rabbit serum) was included in each plate. 1:5000 dilution HRP-conjugated goat anti-rabbit IgG was used as a secondary antibody. Color was developed with 3,3′,5,5′- tetramethylbenzidine (TMB) and the reaction was stopped by 2 M H_2SO_4. The $OD_{450\,nm}$ was measured in a microtiter plate-reader (Bio-Rad Benchmark Plus). The cutoff value was determined when the $OD_{450\,nm}$ ratio ($OD_{450\,nm}$ of purified antibody/$OD_{450\,nm}$ of unimmunized rabbit serum) was above 2.1. Antibody titer was defined as the highest dilution giving a net $OD_{450\,nm}$ value greater than the calculated cutoff.