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Selection and characterization of single-chain recombinant antibodies against spring viraemia of carp virus from mouse phage display library

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Antibody-displaying phage library was selected after three rounds of panning against spring viraemia of carp virus (SVCV) by phage display technology. Eight positive clones which could produce soluble single-chain fragment variable (scFv) antibody induced by isopropyl-beta-D-thiogalactopyranoside (IPTG) were obtained. Dot blot results showed that the eight scFv antibodies could recognize SVCV. The soluble scFv antibodies showed a molecular weight 29 kD by Western blot. All scFv antibodies could recognize SVCV proteins specifically without cross-reaction with other virus proteins by ELISA. Indirect immunofluorescence results showed that all of these scFv antibodies reacted positively with virus in the SVCV-infected cells. These scFv antibodies will be useful tools to establish immunological detection methods for SVCV.

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1. Introduction

Spring viraemia of carp (SVC), a severe viral disease of cyprinid fish, is caused by spring viraemia of carp virus (SVCV). Significant mortality caused by SVC in juvenile carp can reach up to 70% during outbreaks occurring in the spring with water temperatures between 10 and 18 °C (Ahne et al., 2002). SVC has been listed as a notifiable disease by the World Animal Health Organition (OIE) because of its significant risk and harmfulness (Ahne et al., 2002; Liu et al., 2008).

SVCV exhibits a typical bullet shape about 80–180 nm in length and 60–90 nm in diameter and is classified as a member of the family *Rhabdoviridae*, belonging to the genus *Vesiculovirus* (Ahne et al., 2002; Garver et al., 2007). SVCV contains a linear, negative-sense and single-stranded RNA genome. The genomic RNA is 11,019 bases in length. It contains 5 major open reading frames (ORFs) which encodes 5 structural proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and viral RNA-dependent RNA polymerase (L) in the order 3′-N–P–M–G–L–5′ (Bjorklund et al., 1996; Hoffmann et al., 2002; Teng et al., 2007).

Phage display technology is based on recombinant DNA methods allowing co-selection of recombinant antibodies and their respective genes (Wittrup, 1999). If DNA fragments encoding polypeptides are fused to certain bacteriophage coat protein genes, they can be encapsulated within phage particles that also display the encoded polypeptides on their surfaces (Sidhu, 2000; Wong et al., 2001). Therefore, by using recombinant DNA technology, collections of billions of peptides, protein variants, gene fragment-or cDNA-encoded proteins presented on phage (so-called phage-displayed libraries) can be constructed and surveyed for specific affinity and activity. The major advantage of this technological advance is that antibodies could be expressed both on the surface of the phage (antibodies linked to the phage) and in the medium (soluble antibodies) (Gourdine et al., 2005). Q2 In addition, it can be expressed in large amounts in bacterial hosts without the need to immunize animals. So phage display is a powerful technology for selecting and engineering polypeptides with novel functions and has been used widely to produce antibodies. This technique has been applied to the isolation of anti-viral antibodies, such as human cytomegalovirus (HCMV; Takekoshi et al., 1998), white spot syndrome virus (WSSV; Dai et al., 2003) and venezuelan equine encephalitis virus (VEEV; Kirsch et al., 2008). Q3

Since SVCV has a significant impact on carp, the development of a rapid and sensitive method for the detection of it is required urgently. At present, there are many molecular biology detection methods available, but rare for clinical detection. PCR technology has high sensitivity, but it suffers from false positive rate, and is not conformed to recommended by OIE. OIE recommends

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diagnostic tests for SVCV was indirect immunofluorescence and enzyme-linked immunosorbent assay (ELISA) based on monoclonal antibodies (Oreshkova et al., 1999). Although monoclonal antibodies can be generated by a modified hybridoma technology, the entire procedure is extremely complicated, highly expensive and depends on human expertise. So a better method should be established to produce monoclonal antibodies.

In this study, single-chain fragment variable (scFv) antibody against SVCV was panned out from phage antibody library. The aim is to develop a rapid detection method for SVCV. At the same time we hope this work could serves as the basis for further development of a lower cost and simpler detection kit for SVCV.

2. Materials and methods

2.1. Virus and phage display library

The viral pathogen SVCV A-1 was isolated originally from virus-infected common carp in China and preserved in our laboratory (Teng et al., 2007). Viral haemorrhagic septicemia virus (VHSV) strain J167 and infectious haematopoietic necrosis virus (IHNV) were gifted by Centre for Environment, Fisheries and Aquaculture Science (CEFAS) of UK. Pike fry rhabdovirus (PFRV), soft-shelled turtle iridovirus (STIV) and viral nervous necrosis virus (VNNV) strain SSS0607 were isolated and preserved in our laboratory. The cell lines of epithelioma papulosum cyprini (EPC) were preserved in our laboratory. The phage-displayed naïve mouse antibody library was provided by Professor Dai Heping of Institute of Hydrobiology, Chinese Academy of Sciences.

2.2. Panning

The antibody library was screened by three rounds of panning against SVCV, as described by Zhao et al., 2010. The transformed *E. coli TG1* cells were infected with M13KO7 helper phage to rescue the phagemid with its scFv insert. When $OD_{600\,nm}$ value of the library incubated at 37 °C reached 0.5–0.8, M13KO7 helper phage were added to the culture at a multiplicity of infection (MOI) of 5:1 and incubated for 1 h followed by centrifugation and resuspending the cells in fresh 2YT-AK medium (2YT medium containing 100 μg/ml ampicillin and 50 μg/ml kanamycin). After overnight cultivation, the rescued phage was precipitated by addition of PEG/NaCl (PEG8000 in 2.5 M NaCl (20% w/v)) for 1 h on ice bath and collected by centrifugation. The phage was resuspended in 5 ml 2YT medium. Panning of the phage scFv library were performed against SVCV which was coated on immun tube at a concentration of 50 μg/5 ml PBS overnight at 4 °C. After 3 rinses with PBS, immun tube was blocked with 10% skim milk in PBS at 37 °C for 1 h to cover any unoccupied sites. Then prepared phage were added and incubated at 37 °C for 2 h. Unbound phage was removed by 20 rinses with PBST (PBS containing 0.1% Tween-20) followed by 20 rinses with PBS. The SVCV-binding phage was eluted from the immunotube with 1 ml of 100 mM triethylamine followed by neutralization with 0.5 ml of 1 M Tris–HCl (pH 7.5). The eluted phage was then used to reinfect *E. coli TG1* cells at 37 °C for 1 h and then plated onto a SOBAG (SOB containing 100 μg/ml ampicillin and 2% glucose) plate and incubated at 30 °C overnight. Then the first round of panning was over. The titres of input and output phage were calculated for each panning round according to the numbers of colonies on petri dishes of series dilution. Three rounds of panning were performed according to above protocol.

2.3. Selection of antibodies

After three rounds of panning, single colonies were picked up and cultured in 2YT-AG medium (2YT medium containing 2% glucose and 100 μg/ml ampicillin) overnight at 30 °C and 250 rpm, using 96-microwell plates. After that, the bacteria were cultured in 2YT-AK medium supplemented with 1×10^{10} PFU/ml M13KO7 overnight at 30 °C and 250 rpm. The supernatants of the cultures were collected after centrifugation and checked for specificity by ELISA utilizing HRP-anti M13IgG as second antibody.

2.4. DNA sequencing

Nine positive clones with the highest ELISA signal from the third round of panning were selected. Each colony was grown in 2YT-AG medium, and cultures were incubated overnight at 37 °C and 250 rpm. Nucleotide sequence of the positive clones was determined by Beijing Genomic Institute (BGI) Biotechnology Company Limited, Shenzhen, China.

2.5. Production of soluble antibody fragments

After DNA sequencing, positive clones were grown in large volumes overnight for further characterization. The cultures were diluted 1:100 in a fresh 2YT-AG and shaken at 30 °C for 3–4 h until $OD_{600\,nm}$ reached about 0.8. After centrifugation at 4000 rpm for 20 min, the bacterial cells were collected and the supernatant was removed. The bacteria were then resuspended in 2YT-AI (2YT medium containing 100 μg/ml ampicillin and 1 mM IPTG). The cells were grown overnight at 30 °C. After induction, the cells were removed by centrifugation and scFv antibodies were stored at −20 °C until use.

2.6. Characterization analysis of antibody fragments

2.6.1. Dot blot and western blot analysis

The indicated quantities of SVCV, cell debris and PBS were spotted on nitrocellulose membrane. The membrane was then blocked with 3% bovine serum albumin (BSA) in PBS at 37 °C for 1 h. Diluted scFv antibodies were incubated with coated SVCV on the nitrocellulose membrane at room temperature for 1 h. After washing, the binding of the first antibody was assayed with the mouse monoclonal antibody of HRP/Anti-E Tag conjugate, and then its substrates, 3,3′-diaminobenzidine (DAB) were used for colour development.

The soluble scFv antibodies were subjected to SDS-PAGE (12%) and then transferred to a polyvinylidene fluoride (PVDF) membrane as described by Bhaskaran et al., 2005. The membrane was then blocked in 3% (w/v) BSA in PBS for 1 h at room temperature on a rocker. The membrane was washed for three times, 5 min for each with PBS and PBST. The membrane was incubated with the mouse monoclonal antibody of HRP/Anti-E Tag conjugate diluted 1:5000 in PBS containing BSA for 1 h at room temperature. Finally, DAB were used for colour development.

After that, SVCV and cell debris were subjected to SDS-PAGE (12%) and were then transferred to a PVDF membrane. Expressed soluble scFv antibodies as first antibody were detected by Western blot.

2.6.2. Determination of scFv affinity constants

The affinity constant (K_{aff}) of scFv antibodies for SVCV coated onto the 96-well microplate was determined using the protocol described by Beatty et al., 1987. The method is based upon the law of mass action and using solid-phase noncompetitive enzyme immunoassay curves for quantification of antibody content: $K_{aff} = (n-1)/2(n[Ab2]-[Ab1])$ where [Ab1] and [Ab2] represent the respective scFv concentrations required to achieve 50% of the maximum absorbance obtained at two different concentrations of coated antigen ($[Ag]1 = n[Ag]2$), and n is the dilution factor between the two concentrations of antigen used. Affinity constant

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