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Efficacy of a formalin-killed cell vaccine against infectious spleen and kidney necrosis virus (ISKNV) and immunoproteomic analysis of its major immunogenic proteins

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ABSTRACT

Infectious spleen and kidney necrosis virus (ISKNV), the type species of genus *Megalocytivirus* in the family Iridoviridae, is the most important etiological agents in mandarin fish industry in China. Since its first occurrence in China in the early 1990s, there is no effective method to prevent and control this virus. Here we report the successful development of a formalin-killed cell-cultured (FKC) vaccine again ISKNV. Immunoprotection experiments showed that greater than 90% of fish immunized with the FKC vaccine were protected against virulent ISKNV. Sera derived from the immunized fish markedly inhibited the virus infection both *in vitro* and *in vivo*. Purified IgM from the immunized fish sera also showed efficient neutralization effects *in vivo*, strongly suggesting that antibody-mediated immunity may play an important role in the FKC vaccine. Using FKC-immunized fish sera as first antibody, a two-dimensional gel electrophoresis mass spectrometry analysis-based immuno-proteomic method was performed to identify the immunogenic proteins. ORF006L (the major capsid protein), ORF054L, ORF055L, ORF101L, ORF117R, and ORF125R were found to be the major immunogenic proteins of ISKNV. Antibodies generated from these six recombinant viral proteins were able to recognize specifically the corresponding protein fractions from purified virions by Western blot analysis, and five of them (excluding ORF125) showed positive reactions by indirect immunofluorescence assay. In summary, the present study first developed an effective vaccine against ISKNV, and the major immunogenic proteins of ISKNV are also identified. The reported ISKNV immunogenic proteins are important for further development of diagnostic regents and genetic vaccine candidates for all megalocytivirus.

1. Introduction

Megalocytivirus, an emerging piscine iridovirus, has been confirmed as one of the most important causative agents in aquaculture industry in the past decade (Kurita and Nakajima, 2012). Megalocytivirus causes a wide variety of disease in freshwater and marine food fish as well as some ornamental fish, and has attracted increasing attention due to the important economic and ecological significance of these host fish species (Kurita and Nakajima, 2012). Megalocytiviruses that originate from freshwater mainly include mandarin fish *Siniperca chuatsi* infectious spleen and kidney necrosis virus (ISKNV) in

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mainland China (He et al., 2000), murray cod *Maccullochella peelii peelii* iridovirus in Australia (Go et al., 2006), and some small-size tropical ornamental fish iridoviruses in East and Southeast Asian countries (Jeong et al., 2008; Sudthongkong et al., 2002a). The marine fish hosts of megalocytiviruses include many economically valuable cage-cultured fish species in Southeast Asia, such as red sea bream *Pagrus major* (Nakajima and Kunita, 2005), rock bream *Oplegnathus fasciatus* (Do et al., 2004), spotted knifejaw *Oplegnathus punctatus* (Dong et al., 2010), numerous kinds of groupers (Chao et al., 2004; Lü et al., 2005; Ma et al., 2012; Mahardika et al., 2004; Sudthongkong et al., 2002b), flounder *Paralichthys olivaceus* and turbot *Scophthalmus maximus* (Do et al., 2005; Kim et al., 2005; Shi et al., 2004), and many other marine fish species (Gibson-Kueh et al., 2004; Kawakami and Nakajima, 2002; Wang et al., 2007). Molecular epidemiology investigations have indicated that ISKNV-like megalocytiviruses can infect over 50 cultured and wild marine fish species in the South China sea (Wang et al., 2007). In Japan, over 30 cultured marine species are documented as sensitive host fish species to red sea bream iridovirus (RSIV) (Kawakami and Nakajima, 2002). Most recently, megalocytivirus outbreaks have also been associated with the three-spined stickleback *Gasterosteus aculeatus*, a wild temperate fish in North America, indicating that this virus has transmitted outside the traditional area around the Pacific region of Asia (Marcos-López et al., 2011; Waltzek et al., 2012). Among these viruses, ISKNV is defined as the type species of the genus *Megalocytivirus* because it was the first megalocytivirus for which the full genomic sequence was published (Chinchar et al., 2005).

The development of an effective vaccine against these viruses is important in preventing and controlling megalocytivirus-associated diseases. In Japan, a whole-cell vaccine for RSIV has been developed and commercially applied in the marine aquaculture industry (Caipang et al., 2006a; Nakajima et al., 1999). A few megalocytiviral genes have also been selected to develop genetic vaccines, and some of them have shown certain protective effects (Caipang et al., 2006b; Shimmoto et al., 2010; Tamaru et al., 2006). However, the RSIV vaccine is not always suitable for some fish species such as genus *Oplegnathus*, which is highly susceptible to RSIV infection (Kurita and Nakajima, 2012). Poor protective effects have also been recorded among different RSIV stains (Shinmoto et al., 2009).

In the past several years, great advancements have been made in studies of megalocytiviruses. Thus far, the complete genomes and their detailed open reading frame (ORF) annotations of five strains of megalocytiviruses (ISKNV, RSIV, RBIV, OSGIV, and TRBIV) have been determined and published successively since 2001 (Do et al., 2004; He et al., 2001; Kurita and Nakajima, 2012; Lü et al., 2005; Shi et al., 2010). In China, we established a highly susceptible cell line (MFF-1) suitable for propagating both freshwater-borne ISKNV and marine-borne RSIV. Due to its high viral titer for both ISKNV and RSIV, the MFF-1 cell line has great potential for producing a cell-cultured vaccine (Dong et al., 2008, 2010). MFF-1 cells have also been widely used to study the functional genes of ISKNV (Guo et al., 2012; Xu et al., 2010a,b, 2011). The MFF-1 cell platform has also importantly enabled the determination and characterization of the global structural proteins of ISKNV by comprehensive proteomic approaches (Dong et al., 2011). The antigenic profiles of ISKNV proteins have also been primarily analyzed (Xiong et al., 2011). Here, we report a formalin-killed cell-cultured (FKC) vaccine of ISKNV was developed with MFF-1 cells and discuss the protective mechanism. The major immunogenic proteins of ISKNV are determined and characterized using a two-dimensional gel electrophoresis (2DE) based immunoproteomic approach. The reported ISKNV immunogenic proteins are important for further development of diagnostic reagents and genetic vaccine candidates for all megalocytivirus.

2. Materials and methods

2.1. Virus, cell line, and antibodies

MFF-1 cell line were developed and kept in our laboratory (Dong et al., 2008). ISKNV strain NH060831 was previously isolated from ISKNV-infected mandarin fish in 2006, propagated in MFF-1 cells and stored in −80 °C until use (Dong et al., 2008). MFF-1 grown in complete Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Gibco, USA), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Invitrogen, USA) at 27 °C. Rabbit anti-rORF006, anti-rORF054, anti-rORF055, anti-rORF101, anti-rORF117, and anti-rORF125 antibodies were prepared and maintained in our laboratory (Dong et al., 2011). Briefly, ISKNV-ORF006, -ORF054, -ORF054, -ORF055, -ORF101 and ORF125 were cloned into a prokaryotic expression vector, pET-32a and expressed in *Escherichia coli* BL21. The recombinant viral proteins were purified by affinity chromatography on nickel–nitrilotriacetic acid (Ni–NTA) Superflow resin (Qiagen, Germany) according to the manufacturer's instructions. Anti-sera were prepared by immunization of New Zealand white rabbits with the above mentioned six purified recombinant viral proteins, according to a normal operation (Dong et al., 2011). Mouse anti-mandarin IgM monoclonal antibody (7F12F6) was prepared by Wang et al. (2006), and kindly gifted by Dr Hui Gong at Biotechnology Institute, Fujian Academic of Agricultural Sciences, China.

2.2. Preparation of FKC vaccine

Confluent MFF-1 cells were grown in 150 mm^2 tissue culture dishes and inoculated with ISKNV-NH060831 with a multiplicity of infection (MOI) of 1. After 4–5 days, CPE advanced to involve the entire monolayer. Dishes with whole cell suspensions were transferred into −80 °C and kept for at least 24 h. After thawing at room temperature (RT), the cell suspensions were collected into sterile flasks. Viral titer was determined by $TCID_{50}$ assay as described earlier (Reed and Muench, 1938). Formalin with a final concentration of 0.1% (v/v) was added to the cell suspension and mixed thoroughly. After keeping at RT for 24 h, the cell suspensions were transferred to 4 °C for