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Preliminary study of an oral vaccine against infectious hematopoietic necrosis virus using improved yeast surface display technology

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ABSTRACT

Infectious hematopoietic necrosis virus (IHNV) is a common pathogen that causes severe disease in the salmonid aquaculture industry. Because oral vaccines induce more efficient mucosal immunity than parenteral immunization, an oral vaccine was developed with an improved yeast cell surface display technology to induce an immune response to IHNV. The oral yeast vaccine, designated EBY100/pYD1-bi-G, was delivered orally to rainbow trout (*Oncorhynchus mykiss*) on days 1 and 32, and the nonspecific and specific immune responses were measured 50 days after the first vaccination. In the hindgut, spleen, and head kidney, the expression of IFN-1 and Mx-1 was significantly upregulated after oral vaccination with EBY100/pYD1-bi-G, and the highest expression of IFN-1 and Mx-1 was observed in the spleen (7.5-fold higher than the control group) and head kidney (3.9-fold higher than the control group), respectively. Several markers of the adaptive immune response (IgM, IgT, CD4, and CD8) were also significantly upregulated, and the highest expression of these markers was observed in the hindgut, suggesting that the mucosal immune response was successfully induced by oral vaccination with EBY100/pYD1-bi-G. Sera from the orally vaccinated rainbow trout showed higher anti-IHNV neutralizing antibody titers (antibody titer 81 ± 4) than the control sera (antibody titer 7 ± 3), and the relative percentage survival after IHNV challenge was 45.8% compared with 2% in the control group. Although the protection afforded by this orally delivered vaccine was lower than that of a DNA vaccine (83%–98%), it is a promising candidate vaccine with which to protect larval fish against IHNV, which are most susceptible to the virus and difficult to inject with a DNA vaccine.

1. Introduction

Aquaculture is a major global industry, contributing to the national economies of countries with large coastlines, including many countries in South America and Asia (Dhar et al., 2014). However, outbreaks of acute viral diseases are recognized as a major obstacle to aquaculture. Infectious hematopoietic necrosis virus (IHNV) is a common pathogen that causes severe disease and huge economic losses in the salmonid aquaculture industry (Ballesteros et al., 2015). Outbreaks of infectious hematopoietic necrosis (IHN) usually cause 90%–100% mortality, depending on the species and size of the fish (Breyta et al., 2013; Enzmann et al., 2005; Xu et al., 2014). IHNV is a nonsegmented, single-stranded, negative-sense RNA virus belonging to the genus *Novirhabdovirus* in the family *Rhabdoviridae* (Ballesteros et al., 2015). Its RNA is a noninfectious negative-sense molecule that encodes six proteins, including a surface glycoprotein (G) (Anderson et al., 2008; He et al., 2013). Previous studies have shown that the G protein induces a neutralizing antibody response to IHNV (Anderson et al., 1996; Engelking and Leong, 1989; LaPatra et al., 2008; Salonius et al., 2007).

Most IHN vaccines so far reported have been inactivated virus vaccines (Anderson et al., 2008), attenuated vaccines (Fryer et al., 1976; Ristow et al., 2000), and DNA vaccines (Anderson et al., 2008; LaPatra et al., 2001). These are mainly administered by intraperitoneal injection or intramuscular injection. This delivery method is labor intensive and impractical for large-scale administration in the freshwater commercial aquaculture industry because the cost is high (Plant and Lapatra, 2011). Several researchers have reported that the nasal vaccination of rainbow trout induced an

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effective mucosal immune response to IHNV (Salinas et al., 2015; LaPatra et al., 2015), and these results indicate the feasibility of mucosal vaccination. The oral delivery of a vaccine is another appropriate and convenient way to immunize large numbers of small farmed fish, but only a few studies of oral IHNV vaccines have been reported (Ballesteros et al., 2015). The advantages of an oral vaccine include its safety, easy application, limited stress effects, and the less expense, time, and labor required (Adelmann et al., 2008; Rombout et al., 2011).

One of the best approaches to the development of an oral vaccine is to express the virulence protein of the pathogen on the surfaces of *Saccharomyces cerevisiae* cells. *Saccharomyces cerevisiae* has many advantages in the production of an oral vaccine, including its generally recognized safety, easy culture, cheap production, and adjuvant functions (Schreuder et al., 1996a; Schreuder et al., 1996b; Ueda and Tanaka, 2000; Kondo and Ueda, 2004). In previous studies, the yeast surface display system has been used in the design of oral vaccines and for antigen display. Yeast-surface-displayed antigens are potentially efficient vaccines for highly pathogenic avian influenza virus hemagglutinin and *Vibrio harveyi* (Li et al., 2015; Tamaru et al., 2006; Wasilenko et al., 2010). Other oral vaccines have also been proposed by Schreuder and Shibasaki (Schreuder et al., 1996b; Shibasaki et al., 2013). Most of these studies have generated very specific but weak antibody responses, and the most likely explanation for this weak response is the low concentration of antigenic protein on the yeast cell surface. Although the antigens were readily detected, the protein concentrations may not have been high enough to elicit a robust immune response (Schreuder et al., 1996b).

In this study, an oral yeast vaccine containing the glycoprotein (G) of Chinese IHNV strain Sn1203 was developed using an improved yeast display technology. The oral administration of this mucosal vaccine to rainbow trout induced both innate and adaptive immune responses. The vaccine induced high titers of neutralizing antibodies, and provided moderated protection against IHNV infection in the rainbow trout. To our knowledge, this is the first report of the use of *S. cerevisiae* cells displaying the IHNV G protein as a potential oral vaccine against IHNV in rainbow trout.

2. Materials and methods

2.1. Ethics statement

The experiments were performed in accordance with the U.K. Animals (Scientific Procedures) Act (Directive 2010/63/EU) for animal experiments, and these guidelines were followed throughout the study.

2.2. Strains, plasmids, and virus

Escherichia coli strain DH5α was used for the manipulation of recombinant DNA and the *E. coli* Rosetta strain was used for prokaryotic protein expression. *Saccharomyces cerevisiae* strain EBY100 (Trp^-, Leu^-) and the pYD1 plasmid were originally from Invitrogen Life Technologies (V835-01, Carlsbad, California, USA). The pYD1-G and pET-27b-Aga2p-G vectors were constructed in our laboratory, and the glycoprotein gene sequence was submitted to the GenBank database (accession no. KC660147). IHNV strain Sn1203 (KC660147) was used as the challenge virus and the antigen in all the immunoassays performed in the study.

2.3. Construction of yeast oral vaccine

Saccharomyces cerevisiae strain EBY100 was transformed with the recombinant plasmid pYD1-G using electroporation, with a previously described method (Benatuil et al., 2010). YNB-CAA medium containing 20 g/l glucose was inoculated with a transformant containing the expected plasmid and grown overnight at 30 °C with shaking. The cells were collected, resuspended in YNB-CAA medium containing 20 g/l galactose at an optical density at 600 nm (OD_{600}) of 1.0, and grown at 25 °C for 48 h with shaking to display the yeast-derived G protein on the yeast cell surface. These yeast cells were designated EBY100/pYD1-G (only yeast-derived G displayed). *Saccharomyces cerevisiae* strain EBY100 transformed with the pYD1 plasmid was used as the control and designated EBY100/pYD1 (no G protein displayed).

To express the *E. coli*-derived G protein, the *E. coli* Rosetta strain was transformed with the pET27b-Aga2p-G plasmid, and successful transformants were selected by their resistance to kanamycin. Protein expression was induced with isopropyl-β-D-thiogalactoside (IPTG) when the OD_{600} of the cells reached 0.4, and the cells were then cultured for an additional 5 h. The *E. coli*-derived G protein was preliminarily denatured with buffer A (8 M urea, 10 mM Tris, 100 mM NaH_2PO_4, pH 8.0) for 4 h and then renatured for 12 h with buffer B (2 M urea, 10 mM Tris, 100 mM NaH_2PO_4, 1 mM dithiothreitol, 1 mM glutathione [GSH], 2 mM oxidized glutathione [GSSG], pH 8.0). After the protein was renatured, it was purified with ion-exchange chromatography with a sodium chloride gradient (Rustandi et al., 2016). The purified *E. coli*-derived G protein was stored at 4 °C in phosphate-buffered saline (PBS) until required.

To display the *E. coli*-derived G protein on the yeast cell surface, it was incubated with EBY100/pYD1-G yeast cells (which already displayed yeast-derived G after their incubation in galactose medium for 48 h) under oxidizing incubation conditions: in PBS (pH 8.0) containing 1 mM GSH, 0.2 mM GSSG, and 5% dimethyl sulfoxide (DMSO). The yeast cells were incubated for 6 h at 4 °C with stirring, collected, and designated EBY100/pYD1-bi-G (yeast- and *E. coli*-derived G displayed). The cells were then evaluated with western blotting and cellular immunofluorescence.

2.4. Western blotting

The yeast cells collected from the cultures under oxidizing incubation conditions were centrifuged at 1500 × *g*. Protein extracts were prepared by resuspending the cells in 0.1 M NaOH. The cells were incubated at room temperature for 5 min and centrifuged at 10,000 × *g*. The pellet was resuspended in 250 mM Tris (pH 6.8), 140 mM sodium dodecyl sulfate (SDS), 30 mM bromophenol blue, 27 μM glycerol, and 0.1 mM dithiothreitol, as previously described (Andreu and Del Olmo, 2013). After incubation at 95 °C for 5 min, the samples were centrifuged for 10 min at 1500 × *g*, and the supernatants were analyzed on SDS-PAGE gels and then with western blotting. A rabbit Anti-Xpress™ tag antibody (ab1257, Abcam, United Kingdom) was used to probe the western blots.

2.5. Cell immunofluorescence analysis

Cells were collected and washed three times with PBS. A rabbit anti-6×His tag antibody (ab125262, Abcam) and a mouse anti-hemagglutinin (HA) antibody (ab18181, Abcam) were both used as primary antibodies. A fluorescein isothiocyanate (FITC)-tagged anti-rabbit IgG antibody (ab6717, Abcam) and a Cy3-tagged anti-mouse IgG antibody (ab97035, Abcam) were used as the secondary antibodies. The samples were washed three times with PBS and analyzed with fluorescence microscopy (Leica, DMi8, Wetzlar, Germany).

2.6. Oral vaccination of rainbow trout

Rainbow trout (5 ± 1 g mean weight) were purchased from a local spring-water farm with no previous record of IHNV outbreaks