Fish & Shellfish Immunology 31 (2011) 212–216

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Oral immunization of olive flounder (*Paralichthys olivaceus*) with recombinant live viral hemorrhagic septicemia virus (VHSV) induces protection against VHSV infection

Min Sun Kim [a], Dong Soo Kim [b], Ki Hong Kim [a,*]

[a] Department of Aquatic Life Medicine, Pukyong National University, Nam-gu 599-1, Busan 608-737, South Korea
[b] Department of Marine Bio-materials & Aquaculture, Pukyong National University, Busan 608-737, South Korea

ARTICLE INFO

Article history:
Received 15 March 2011
Received in revised form
9 May 2011
Accepted 9 May 2011
Available online 17 May 2011

Keywords:
Recombinant VHSV
rVHSV-Δ
NV-EGFP
Live vaccine
Oral vaccine

ABSTRACT

A recombinant viral hemorrhagic septicemia virus (rVHSV-ΔNV-EGFP) that has enhanced green fluorescent protein (EGFP) gene instead of NV gene was previously generated using reverse genetics technology. In this study, potential of the rVHSV-ΔNV-EGFP to be used as a live oral vaccine candidate was assessed. The presence of the recombinant virus in internal organs of orally administered olive flounder (*Paralichthys olivaceus*) was analyzed by semi-quantitative RT-PCR. Although the recombinant VHSV-specific band was detected only when the number of PCR cycle was increased to 35, the band was detected from internal organs, such as kidney, spleen, and liver of fish that were reared at either 15 °C or 20 °C till even 20 days, suggesting that a few orally administered rVHSV-ΔNV-EGFP might be transported to internal organs, and might keep weak replication ability in the organs. VHSV-neutralizing activity was induced by oral immunization of olive flounder with the NV gene knock-out recombinant VHSV not only in skin and intestinal mucus but also in serum, suggesting that mucosal and systemic adaptive immune responses were elicited by oral immunization. In challenge experiment, groups of fish immunized with 10^4, 10^5, and 2×10^5 PFU of rVHSV-ΔNV-EGFP/fish showed 25%, 50%, and 70% of relative percent survival (RPS), respectively. The RPSs were elevated to 60%, 75%, and 90% by a boost immunization in fish boost immunized with 10^4, 10^5, and 2×10^5 PFU of rVHSV-ΔNV-EGFP, respectively. The cumulative mortality of fish in the control groups was 100%. Conclusionly, the present results demonstrate that the NV gene knock-out recombinant VHSV administered orally to olive flounder can induce dose- and boosting-dependent VHSV-neutralizing antibody in mucus and serum, and can provide a high protection in olive flounder against a virulent VHSV challenge.

1. Introduction

Viral hemorrhagic septicemia disease (VHSD) caused by viral hemorrhagic septicemia virus (VHSV), an enveloped negative-strand RNA virus belonging to the genus *Novirhabdovirus* of the family Rhabdoviridae [1–3], is regarded as one of the most economical important disease of cultured fish world-wide because of resulting in high mortalities [4–7]. In the genome of VHSV, genes encoding five structural proteins; a nucleoprotein (N), a polymerase-associated phosphoprotein (P), a matrix protein (M), a glycoprotein (G), an RNA-dependent RNA polymerase (L), and a nonstructural protein (NV) are arranged in the order 3′-N-P-M-G-NV-L-5′ [8]. VHSV is currently subdivided into four major genotypes (I, II, III, and IV) based on the genetic analysis of G and N genes [9,10], and virulence of each genotype was different according to host fish species [11,12]. To date, only the genotype IVa of VHSV has been reported in Korea, and outbreaks of VHSD have brought severe loss in olive flounder, *Paralichthys olivaceus*, aquaculture [13,14].

As no effective anti-VHSV chemotherapeutic agents are available, development of effective vaccines would be a way to prevent spreading of VHSD in aquaculture farms. Several types of prophylactic vaccines against VHSV have been reported, such as subunit vaccine [15,16], naturally attenuated vaccine [17–19], and genetic vaccine [20–24]. Especially, Byon et al. [25,26] reported on the protective effect of DNA vaccine and subunit vaccine against VHSV in olive flounder. Recently, the effectiveness of live recombinant infectious hematopoietic necrosis virus (IHNV) vaccines that were made by reverse genetics method has been reported [27–29]. However, there were no reports on the vaccine potential of

* Corresponding author. Tel.: +82 51 629 5943; fax: +82 51 629 5938.
E-mail address: khkim@pknu.ac.kr (K.H. Kim).

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doi:10.1016/j.fsi.2011.05.003

recombinantly attenuated VHSVs except a paper lately reported from our laboratory [30].

To avoid injection-related risks and high labor costs, an oral vaccine is clearly a more acceptable alternative, and may be more suitable way to immunize small fingerlings. It is well-known in mammals that vaccines administered at mucosal surfaces can induce not only mucosal immunity that provide local protection against mucosally transmitted pathogens but also systemic immunity [31–33]. In spite of these advantages, mucosal immunization with inactivated or subunit vaccines generally showed low ability to induce immune responses and had high probability to induce immunological tolerance [33]. Vaccines made with live pathogens would provide an effective way to overcome the weaknesses of mucosal vaccines.

In our earlier reports, we presented the results of a generation of a recombinant VHSV (rVHSV) by changing NV gene ORF with EGFP gene ORF (rVHSV-ΔNV-EGFP) (submitted for publication), and a high protection of olive flounder against VHSV infection by intramuscular immunization with the recombinant VHSV [30]. In this study, we assessed the potential of the rVHSV-ΔNV-EGFP to be used as a live oral vaccine candidate in olive flounder. The results of the present study showed that the NV gene knock-out VHSV induced protective immunity following oral vaccination of olive flounder.

2. Materials and methods

2.1. Virus and cell line

VHSV KJ2008 (wild-type VHSV) used in this study was isolated in 2008 from moribund olive flounder in a natural outbreak of VHS disease on a commercial farm in Korea. The genotype of VHSV KJ2008 was identified as IVa by sequencing of the N gene and G gene. The recombinant virus, rVHSV-ΔNV-EGFP, was generated by reverse genetics using genome of VHSV KJ2008 as the template (the procedures for rescue of recombinant VHSV are not shown here). The viruses were propagated in monolayer of *Epithelioma papulosum cyprini* (EPC) cells cultured in Leibovitz medium (L-15, Sigma) at 15 °C in the presence of 2% fetal bovine serum (FBS, Gibco) and antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml). Cultures displaying extensive cytopathic effect (CPE) were harvested and centrifuged 4000 *g* for 10 min at 4 °C, and the supernatants were stored at −80 °C. Plaque assay [34] was used to determine titer of the viruses.

2.2. rVHSV-ΔNV-EGFP load in internal organs of fish

The load of orally administered rVHSV-ΔNV-EGFP in internal organs of olive flounder was analyzed using semi-quantitative reverse transcriptase PCR (RT-PCR). Olive flounder fingerlings (body weight 4–5 g) confirmed free-from VHSV before experiments were acclimated for 2 weeks in 50 L tanks at either 15 °C or 20 °C, and were administered the rVHSV-ΔNV-EGFP at a dose of 2×10^5 PFU/fish through an oral route using gastric tubes. At 6 h, 12 h, 1 d, 2 d, 3 d, 5 d, 7 d, 10 d and 20 d after the oral administration, the spleen, kidney and liver were excised from 3 fish in each group, and total RNA was extracted using RNAiso Plus (Takara) from each organ. The nucleotide sequence of PCR primers used to amplify a region specific for rVHSV-ΔNV-EGFP was as follows: forward primer; 5′- ACCAGAGCATCTATGAC AGCGGAA-3′, reverse primer; 5′-TGGTCGGGGTAGCGG CTGAAG-3′, and for a control 18S ribosomal RNA gene was: forward primer; 5′-CAAGACGGACGAAAGCGAAAGCAT-3′, reverse primer; 5′-TGGCATC

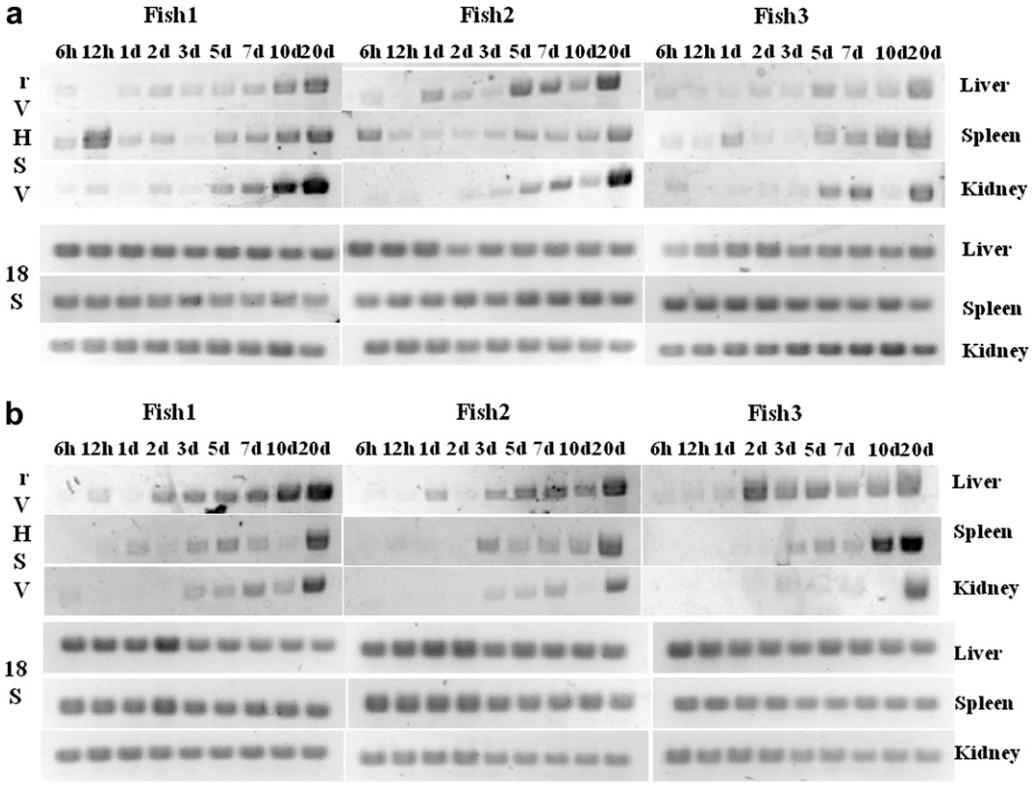

Fig. 1. Load of orally administered recombinant VHSV (rVHSV-ΔNV-EGFP) in internal organs of olive flounder (*Paralichthys olivaceus*) was analyzed using semi-quantitative RT-PCR. Olive flounder reared either at 15 °C (a) or at 20 °C (b) were orally administered with 2×10^5 PFU/fish of rVHSV-ΔNV-EGFP. At 6 h, 12 h, 1 d, 2 d, 3 d, 5 d, 7 d, 10 d, and 20 d after the oral administration, the spleen, kidney and liver were excised from randomly sampled 3 fish in each group, and analyzed the amplification of transcripts corresponding to rVHSV-ΔNV-EGFP specific-region (rVHSV; stretching over VHSV G gene and EGFP gene) and olive flounder's 18S ribosomal RNA gene (18S).