Aquaculture 446 (2015) 259–262

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Short communication

Protective potential of a plasmid having different classes of CpG motifs against viral hemorrhagic septicemia virus and *Miamiensis avidus* (Ciliata; Scuticociliatida) infections in olive flounder (*Paralichthys olivaceus*)

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ARTICLE INFO

Article history:
Received 15 April 2015
Received in revised form 8 May 2015
Accepted 11 May 2015
Available online 14 May 2015

Keywords:
CpG motifs-containing plasmids
Different classes CpGs
Protection
VHSV
Miamiensis avidus
Olive flounder

ABSTRACT

Synthetic oligodeoxynucleotides containing CpG motifs (CpG-ODNs) are potent immunostimulators in vertebrates. Based on the backbone structure and the oligonucleotides sequences, CpG-ODNs can be classified into three different classes (A, B, and C) that induce class-specific immune responses. Plasmids with stimulatory CpG motifs in their backbone also show immunostimulatory effects, and a plasmid-based delivery of CpG motifs can be an alternative approach to expensive CpG-ODNs. In this study, we constructed a plasmid (pL-CpGmix) that harbors multiple copies of CpG-ODN 2216 (class A), 1668 (class B), and 2395 (class C) motifs, and evaluated the potential of the constructed plasmid to protect of olive flounder (*Paralichthys olivaceus*) from viral hemorrhagic septicemia virus (VHSV) and *Miamiensis avidus* infections. Fish were administered pL-CpGmix, pL-GFP that harbored a green fluorescent protein (GFP) gene, empty vector, or phosphate buffered saline (PBS) alone. On 3 days post-administration, fish were challenged with VHSV or *M. avidus*. Fish administered pL-CpGmix showed the lowest cumulative mortality (60%) against a VHSV challenge, which was significant compared to that of the control groups (empty vector and PBS). In the *M. avidus* challenge experiment, fish in the group administered pCpG-mix showed the highest survival rate and serum scuticocidal activity, and those were statistically significant compared to those of control groups. Although fish administered pL-GFP showed lower survival rates than fish administered pL-CpGmix, the GFP-containing vector induced higher survival rates against *M. avidus* and VHSV infections and higher serum scuticocidal activity compared to the control groups, suggesting that GFP sequence might have an immunostimulating property. The present results showed that the plasmid harboring different classes of CpG motifs enhanced resistance of olive flounder against both *M. avidus* and VHSV infections.

1. Introduction

The DNA containing unmethylated CpG dinucleotides is known to have a strong immunostimulatory ability in vertebrates including fish (Klinman, 2004; Tassakka and Sakai, 2005). Due to a low frequency of unmethylated CpGs in the genome of vertebrates, microbial genomic DNA that has relatively high unmethylated CpGs is recognized as a pathogen associated molecular pattern (PAMP) by the toll-like receptor 9 (TLR9) (Krieg et al., 1995; Hemmi et al., 2000; Krieg, 2002, 2003). The TLR9-mediated signal cascades induce diverse immune responses such as production of pro-inflammatory cytokines and type I interferon responses (Aderem and Ulevitch, 2000; Akira and Takeda, 2004).

Synthetic CpG motifs-containing oligodeoxynucleotides (CpG-ODNs) mimicking microbial DNA are also potent immunostimulators in vertebrates (Manuja et al., 2013; Shirota and Klinman, 2014). Based on the backbone structure and oligonucleotides sequences, CpG-ODNs can be classified into three different classes (A, B, and C) that induce class-specific immune responses (Krieg, 2002). In a previous study (Kang and Kim, 2012), we demonstrated that CpG-ODN 2216 (A-class ODN) elicited a strong type I interferon response and induced high resistance against viral hemorrhagic septicemia virus (VHSV) in olive flounder (*Paralichthys olivaceus*), whereas CpG-ODN 1668 (B-class) elicited a significantly high serum scuticocidal activity and a survival rate against *Miamiensis avidus* challenge. Furthermore, CpG-ODN 2395 (C-class) showed abilities intermediate between ODN 2216 and ODN 1668 in inducing resistance against VHSV and *M. avidus*.

In spite of the high immunostimulatory effects of CpG-ODNs, the cost for the synthesis of CpG-ODNs is too high to be widely used in

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http://dx.doi.org/10.1016/j.aquaculture.2015.05.014
0044-8486/

aquaculture farms. Recently, it has been reported that CpG motif-enriched plasmids can be used to modulate immune responses and to enhance protective effects of DNA vaccines (Coban et al., 2005; Hung et al., 2011). As the cost for the production of CpG motif-enriched plasmids is much lower than for the synthesis of CpG-ODNs, a plasmid-based delivery of CpG motifs would be an alternative approach to expensive CpG-ODNs. In fish, it has been demonstrated that plasmids with stimulatory CpG motifs in their backbone had immunostimulatory effects that are ranging from humoral to cellular immune responses (Chen et al., 2007; Liu et al., 2010).

In this study, we postulated that a plasmid containing multiple copies of CpG motifs that are belonging to different classes may stimulate diverse innate immune factors that can confer simultaneous protection against both viral and parasitic pathogens. Thus, we constructed a plasmid that harbors multiple copies of CpG-ODN 2216, 1668, and 2395 motifs, and evaluated the potential of the constructed plasmid to protect from VHSV and *M. avidus* infections in olive flounder.

2. Materials and methods

2.1. Ciliates and VHSV

M. avidus isolated from the brain of a diseased olive flounder (sampled in 2012) were aseptically grown using *Epithelioma papulosum cyprini* (EPC) cells in Leibovitz medium (L-15, Sigma) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% fetal bovine serum (FBS, Gibco) at 20 °C. VHSV KJ2008 isolated from a moribund olive flounder (sampled in 2008) was propagated in the monolayer of EPC cells at 15 °C in the presence of 2% FBS and antibiotics. Cultures displaying extensive cytopathic effect (CPE) were harvested and centrifuged 4000 *g* for 10 min at 4 °C, and the supernatants were filtered with a 0.45 μl syringe filter (Advantec, USA) and stored at −80 °C. The plaque forming unit (PFU) of the VHSV stock was determined according to the method of Burke and Mulcahy (1980).

2.2. Plasmids construction

A fragment corresponding to randomly arranged 6 copies of each CpG-ODN motif (CpG-ODN 1668, 5′-TCCATGACGTTCCTGATGCT-3′; CpG-ODN 2216, 5′-GGGGGACGATCGTCGGGGGG-3′; and CpG-ODN 2395, 5′-TCGTCGTTTTCGGCGCGCGCCG-3′) was artificially synthesized and inserted into pUC57 vector (Cosmo Genetech, Korea). After digestion with *Bgl*II and *BamH*I, the fragment containing multiple CpG motifs was ligated into LITMUS 28i vector (NEB) that was pre-digested with the same restriction enzymes, and designated it as pL-CpGmix. A previously constructed vector, pL-GFP (Kang et al., 2014a), containing the green fluorescent protein (GFP) gene sequence was used as a control vector.

2.3. In vivo experiments

Olive flounder fingerlings weighing approximately 4–5 g were obtained from a local fish hatchery in Korea, and were acclimated at least for 2 weeks before the experiments. Prior to experiments, 10 fish were randomly sampled and the free of VHSV and scuticociliates was confirmed by a PCR analysis for VHSV and a microscopic observation for scuticociliates.

2.3.1. VHSV

Sixty olive flounder fingerlings were divided into four 50 l tanks (15 fish/tank) and placed the tanks in a 1 t aquarium that equipped with a temperature control function. Fish were adapted to 15 °C by gradual decrease of water temperature. Fish were intraperitoneally injected (i.p.) with 100 μg of pL-CpGmix, pL-GFP, or empty vector (pLITMUS 28i). Fish in a control group injected with 50 μl of phosphate buffered saline (PBS) alone. On 3 days post-injection, the fish were intramuscularly (i.m.) challenged with VHSV KJ2008 at 10^3 PFU/fish. The mortality was monitored for 14 days. Relative percent survival (RPS) was calculated as follows (Amend, 1981);

$$\mathrm{RPS} = [1-(\%\ \text{mortality in experimental group}/\%\ \text{mortality in control group})] \times 100\%.$$

2.3.2. M. avidus

Fish were divided into 4 groups with 2 replicates, and kept in eight 50 l tanks (18 fish/tank) at 21–22 °C. Fish were i.p. injected with 100 μg of pL-CpGmix, pL-GFP, empty vector or 50 μl of PBS alone. On 3 days post-injection, 4 fish in each tank were randomly sampled and bled to isolate serum. The rest of fish in one replicate were i.p. challenged with 2×10^4 ciliates, and in the other replicate were challenged with 2×10^5 ciliates. The mortality was monitored for 18 days, and dead fish were necropsied to confirm the presence of ciliates.

2.4. Serum scuticocidal activity

The isolated sera were serially diluted (1/2–1/256) using Hank's balanced salt solution (HBSS, Sigma) and 96-well flat-bottomed plates. The cultured ciliates were added to the wells (1×10^2 ciliates/well) of the plate containing the serially diluted sera, incubated at 20 °C, and observed for 24 h. The scuticocidal titer of each serum was the last dilution at which 100% of the ciliates were lysed or non-motile, which was observed under an inverted microscope at 40–100× magnification. In all assays, control wells containing heat-inactivated serum and HBSS alone were included.

2.5. Statistical analysis

Statistical analysis was performed using SPSS for Windows (SPSS, USA). Data on the serum scuticocidal activity were analyzed by one-way ANOVA followed by Tukey HSD post-hoc test. The Kaplan–Meier method was used to analyze significance of the cumulative mortality. A probability (P) value less than 0.05 was considered statistically significant.

3. Results

3.1. Protection against VHSV

Groups of fish administered PBS alone and empty vector showed 100% cumulative mortalities at 8 and 10 day post-challenge, respectively (Fig. 1). The group administered pL-GFP showed 20% of RPS. The pL-CpGmix-administered group showed the lowest cumulative mortality (40% of RPS), which was significant compared to that of PBS and empty vector administered groups (Fig. 1).

3.2. Protection against M. avidus

The cumulative mortalities of fish in PBS and empty vector administered groups were 100% by a challenge with 2×10^4 or 2×10^5 *M. avidus* (Fig. 2). The group of fish administered pL-GFP and challenged with 2×10^5 ciliates showed 21.5% of RPS, and was significantly different with PBS and empty vector administered groups (Fig. 2B). Fish administered pL-CpGmix showed 35.7% and 28.6% of RPS by challenge with 2×10^4 and 2×10^5 ciliates, respectively, which were statistically significant compared to that of PBS and empty vector administered groups (Fig. 2).

3.3. Scuticocidal activity of serum

Fish administered pL-CpGmix showed the highest serum scuticocidal activity which was significantly higher than that of PBS