Aquaculture 492 (2018) 369–378

Contents lists available at ScienceDirect

Aquaculture

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

Immunogenicity of inactivated formalin-killed *Photobacterium damselae* subsp. *piscicida* combined with Toll-like receptor 9 agonist in Cobia *Rachycentron canadum*

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ARTICLE INFO

Keywords:
Adjuvanticity
Vaccination
Cobia *Rachycentron canadum*
Immune response
Photobacterium damselae subsp. *piscicida*

ABSTRACT

The immune responses and protection efficacy of a vaccine containing inactivated formalin-killed *Photobacterium damselae* subsp. *piscicida* formulated with alum and different unmethylated Cytosine phosphorothioate Guanine oligodeoxynucleotides (CpG-ODN, including 2006, 2395, 1668, and control ODN 2137) as the adjuvant were investigated in cobia *Rachycentron canadum*. Results showed that, formulation with CpG-ODN 1668 significantly ($P < .05$) increased the expression of *rctlr9a* (11 and 20 fold in spleen and liver, respectively) and *il-1β* (4 fold in liver) in immunized fish. The highest lysozyme and peroxidase activities were also observed in fish immunized with CpG-ODN 1668 formulation. In addition, compared to other treatments, bactericidal activity using the bacterial survival percentage was significantly less in CpG-ODN 1668 formulation (25%). The antibody titer was significantly higher at 6 and 21 dpi from CpG-ODN 1668 (OD = 0.27 ± 0.02) and CpG-ODN 2006 (OD = 0.24 ± 0.018) formulations. Fish immunized with CpG-ODN 1668 and 2395 formulations protected *R. canadum* with survival rate of 90 & 70% by *P. damselae* subsp. *piscicida* until 3 weeks post-challenge. This study has indicated that different *tlr9* agonists have different abilities to augment immune response and protection against Photobacterium. CpG-ODN 1668 can be used as an adjuvant for *R. canadum* vaccination.

1. Introduction

In vertebrates, molecular structures in pathogens known as pathogen-associated molecular patterns (PAMPs) are recognized by innate immune cells *via* pattern recognition receptors (PRR) (Gao et al., 2012). The innate immune system has developed the ability to recognize these unmethylated CpG dinucleotides within certain sequence contexts (CpG motifs) in bacterial and some viral DNA (Krieg, 2002) by Toll-like Receptor 9 (*tlr*9) (Krug et al., 2001; Kumagai et al., 2008; Kawai and Akira, 2011). The *tlr*9 molecules of different species have diverged throughout evolution, so that the precise CpG motifs optimal for stimulating cells varies from one species to another (Rankin et al., 2001; Chaung, 2006; Byadgi et al., 2014a).

Further, irrespective of applying CpG-ODN containing DNA to the host, cellular uptake is an essential step (Hartmann and Krieg, 2000; Manzel and Macfarlane, 1999). Uptake of DNA initiates *tlr*9 relocation from the endoplasmic reticulum to the endosome (De Jong et al., 2010; Latz et al., 2004; Sanjuan et al., 2006) where the presence of DNA containing CpG-ODN motifs can be recognized by *tlr*9 (Sanjuan et al., 2006; Hemmi et al., 2000; Rutz et al., 2004). Activated *tlr*9 augments the expression of co-stimulatory molecules, such as major histocompatibility complex (MHC) II (Hartmann et al., 1999), type I interferon (IFN), *il-12*, *il-1β*, *ifn-γ*, *il-6* and tumor necrosis factor (*tnfα*), which contributes to the development of innate and adaptive immune responses (Klinman et al., 1996; Van Uden et al., 2001). The immune responses elicited by CpG-ODNs vary depending on the class of CpG motifs, flanking sequence, length, backbone modification and formation of secondary and tertiary structures (Krieg, 2006). Studies in mice and humans showed three distinct classes (A, B and C) of CpG-ODNs on the basis of difference in their structures and immune stimulating effects (Vollmer et al., 2004a).

CpG-A class ODN have a native phosphodiester palindromic central

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https://doi.org/10.1016/j.aquaculture.2018.04.035
Received 4 July 2017; Received in revised form 10 April 2018; Accepted 20 April 2018
Available online 23 April 2018
0044-8486/

region flanked by nuclease resistant phosphorothioate backbones at both ends, which induces the production of high levels of *ifn*-α from plasmacytoid dendritic cell (pDC) with relatively little B-cell stimulation. CpG-B ODNs are completely phosphorothioate modified and do not form secondary structures (Vollmer et al., 2004a). CpG-B induces the production of low levels of *ifn*-α along with profound B-cell activation (Vollmer et al., 2004b). CpG-C ODNs are also phosphorothioate sequences with a 3′ palindromic permitting the formation of duplexes (Mena et al., 2003). CpG-C ODNs combine strong *ifn*-α induction and efficient pDC maturation and efficiently stimulate B cells. Both CpG-A and CpG-B have been indicated to have immunostimulatory properties in the immune cells of mice, primates and many domestic species *in-vitro* (Rankin et al., 2002; Kamstrup et al., 2001; Pontarollo et al., 2002; Kurata et al., 2004; Guzylack-Piriou et al., 2004; Abel et al., 2005), while *in-vivo* studies in animals have mainly been carried out with CpG-B (Hartmann et al., 2004; Nichani et al., 2004a,b; Reginald et al., 2007). In this study, we used CpG-B and CpG-C ODNs with the exception of CpG-A ODNs, as because CpG-A ODNs are less activators of B-cells and the similar functions could be considered from CpG-B and CpG-C ODNs. Moreover, the immunostimulatory properties of CpG-ODNs are based on human *Homo sapiens* L. 1758 and mouse *Mus musculus* L. 1758. In fish, many efforts have been made to identify the optimal CpG motifs for cross species specificity, but the immune response and disease resistance vary with species and type of CpG-ODNs used for stimulation (Strandskog et al., 2007; Kang and Kim, 2012). The literature shows that there is no universal CpG-ODN for activation of fish immune responses on different type of immune cells. Also, immune cells can vary within the same species.

Cobia *Rachycentron canadum* L. 1766 occupies a distinct position in world aquaculture FAO, 2012). The *R. canadum* farming industry has been suffering a growing damage from various infectious diseases mainly from *Photobacterium damselae* subsp. *piscicida* (Chang et al., 2006; Guo et al., 2006). Photobacteriosis in its acute form, can cause multifocal necrosis in the liver and spleen and bacteria can accumulate freely in phagocytes, capillaries and interstitial spaces (Andreoni et al., 2013). To date, several types of *P. damselae* subsp. *piscicida* vaccines have been reported in different fishes, including inactivated bacteria (Romalde and Magarinos, 1997), LPS formulations in yellowtail *Seriola quinqueradiata* (Temminck & Schlegel, 1845) (Fakuda and Kusuda, 1985), ECP-enriched bacterin preparation in gilthead seabream *Sparus aurata* L. 1758 (Magarinos et al., 1994); water-in-oil emulsion vaccine in *S. quinqueradiata* (Gravningen et al., 2008), subunit vaccine in *R. canadum* and seabass *Dicentrarchus labrax* L. 1758 (Andreoni et al., 2013; Ho et al., 2011). However, no studies on *R. canadum* have been conducted using a formulation of vaccine by CpG-ODNs as an adjuvant on *P. damselae* subsp. *piscicida.* Additionally, the immunostimulatory effects of CpG-ODNs on adaptive immune response can be enhanced by formulating with compounds commonly used in animal vaccines such as alum (Linghua et al., 2006). CpG-ODNs (negatively charged phosphate groups in the backbone) and alum (positively charged) are electrostatically attracted to each other and so can work synergistically (Aebig et al., 2007). In addition to the electrostatic consideration, other mechanisms, including phosphate ligand exchange and hydrophobic interactions, also can attract adjuvants or antigens to the alum (Lindblad, 2004).

*Rachycentron canadum tlr*9 was cloned and its involvement in the immune responses elicited by CpG-ODNs and immunostimulatory effects were investigated based on the gene expression within a short duration after injection (10 dpi) (Byadgi et al., 2014a; Byadgi et al., 2014b). However, the long term adjuvanticity of CpG-ODNs when formulated in an inactivated vaccine was not addressed. Therefore, the objectives of this study were to combine different CpG-ODNs with formalin killed bacterial vaccine containing alum, and to evaluate their effects on 1) *rctlr*9, *myd88* and *il-1β* expression, 2) humoral response in serum and 3) the protection efficacy post bacterial challenge.

2. Materials and methods

2.1. CpG-ODNs

Oligonucleotides were purchased from Bioneer (www.bioneer.com). The ODNs were phosphorothioate modified throughout the sequence. Sequences of ODNs used are:

B- Class CpG 1668 T*C*C*A*T*G*A*C*G*T*T*C*C*T*G*A*T*G*C*T,

B-Class CpG 2006 T*C*G*T*C*G*T*T*T*T*G*T*C*G*T*T*T*T*G*T*C*G*T*T.

C- Class CpG 2395 T*C*G*T*C*G*T*T*T*T*C*G*G*C*G*C*G*C*G*C*C*G and ODN 2137 (control CpG with phosphorothioate modified inverted) T*G*C*T*G*C*T*T*T*T*G*T*G*C*T*T*T*T*G*T*G*C*T*T (phosphorothioate modifications are marked with * and CG and GCs are in bold).

The CpG ODNs were suspended (1 μg/μl) in phosphate buffer saline (PBS, pH 7.2) and stored at −20 °C until use.

2.2. The adjuvanticity of CpG ODNs

2.2.1. Preparation of inactivated formalin killed bacterial (FKB) vaccine

Inactivated bacteria were prepared from the frozen stock using the following conditions: a virulent strain of *Photobacterium damselae* subsp. *piscicida* (Tungkang Biotechnology Research Center, Council of Agriculture, Taiwan) was cultured in 5 ml Brain Heart Infusion (BHI, BD Difco™; www.bd.com) broth containing 2% NaCl at 28 °C for 24 h and then 1 ml broth of the stock was transferred to 100 ml broth and grown until the O.D reached to 1.0 (600 nm, Thermo Fisher Scientific; www.thermofisher.com). Subsequently, calculated the cell concentration, from O.D 1.0 as colony-forming units (CFU)/mL, prior to inactivation and recorded 1×10^6 cells mL^{-1} The bacteria were harvested by centrifugation 6000 ×*g* at 4 °C (Thermo Scientific; www.thermofisher.com) for 10 min. The pellet was washed twice in PBS (pH 7.2), and the bacterial suspension was inactivated by adding formalin to a final concentration of 3% and incubated 12–15 h at 4 °C. The inactivated bacterial solution was centrifuged at 6000 ×*g* for 10 min and thoroughly washed for three times using 5 ml of PBS (pH 7.2) during each centrifugation in order to remove formalin and then re-suspended in PBS adjusting the O.D reached to 1.0 (600 nm, Thermo Fisher Scientific; www.thermofisher.com). The inactivation of formalin killed bacteria (FKB) was confirmed by plating 100 μl of solution on BHI + 2% NaCl agar plates and incubated at 28 °C overnight.

2.2.2. Formulation of FKB, alum and CpG-ODNs

Inactivated vaccines were formulated for individual fish which contain 10 μg of CpG-ODNs separately (1668, 2006, 2395 and 2137 as control) with 45 μl of alum (InvivoGen; www.invivogen.com) and 45 μl of FKB (Table 1). Formulations were mixed at room temperature (25 °C) on a shaker at approximately 100 rpm for 30 min and stored at 4 °C until use.

2.2.3. Immunization and sampling

Rachycentron canadum were procured from a local fish farm in Pingtung, Taiwan and were acclimatized in recirculating aerated tanks for up to one week. During acclimatization they were fed with a commercial diet and proper water quality was maintained (water volume 400 l, temperature 28 °C and salinity 30‰). After acclimatization, thirty fish per tank were stocked into six different groups (Table I). The first group was immunized intraperitoneally (i.p.) with 100 μl PBS. The second group was injected with PBS (10 μl) + FKB (45 μl) + alum (45 μl). The third group was immunized with 100 μl/fish vaccine containing ODN 2137 (10 μg, 1.42 nM) + 45 μl FKB + 45 μl alum, fourth group with 1668 CpG-ODNs (10 μg, 1.57 nM) plus 45 μl FKB and 45 μl of alum, fifth group with CpG-ODN 2006 (10 μg, 1.40 nM) + 45 μl FKB + 45 μl alum, sixth group with CpG-ODN 2395 (10 μg,