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Immunogenicity and efficacy of DNA vaccine encoding antigenic AcfA via addition of the molecular adjuvant Myd88 against *Vibrio alginolyticus* in *Epinephelus coioides*

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ABSTRACT

DNA vaccines had been widely used against microbial infection in animals. The use of molecular adjuvants to improve the immunogenicity of DNA vaccines has been increasingly studied in recent years. MyD88 is one of the adapter molecules to activate the signaling cascades and produces inflammatory mediators, and its immunological role and adjuvant potential which had been proved in mammals were rarely reported in fish species. In this study, plasmid pcMyD88 was constructed and the capacity of MyD88 as molecular adjuvant was explored by co-injecting with a DNA vaccine encoding AcfA against *Vibrio alginolyticus* infection in orange spotted grouper. The results suggested that it needed at least 7 days to transported DNA vaccine pcacfA or molecular adjuvant pcMyD88 from the injected muscle to kidney and spleens and stimulate host's immune system for later protection. The co-injection of pcMyD88 with DNA vaccine pcacfA could increase significantly specific antibody levels and the expression levels of the immune-related genes including MHCIα, MHCIIα, CD4, CD8α, IL-1β and TNFα. Furthermore, pcMyD88 enhanced the immunoprotection of pcacfA against *V. alginolyticus* infection, with the significantly higher RPS of 83.3% in pcMyD88 + pcacfA group compared with that of pcacfA alone (73.3%) at challenging test of 10 weeks post vaccination. Together, these results clearly demonstrate that MyD88 is an effective adjuvant for the DNA vaccine pcacfA in orange spotted grouper.

1. Introduction

Vibriosis is one of the most prevalent diseases causing high mortality in marine aquatic animals [1]. *Vibrio alginolyticus* is a ubiquitous organism in seawater and has been associated with several epidemics of vibriosis in marine animals, including fish, shellfish, crustaceans, and coral reefs [2,3]. In the coastal provinces of South China, *V. alginolyticus* is one of the important causative agents of vibriosis that endangered the aquaculture of marine animals [4,5]. The commercially important orange spotted grouper *Epinephelus coioides* is an economically important cultivated marine species because of its robustness in heavily populated conditions and its rapid growth at elevated temperatures in China [6,7]. *V. alginolyticus* is the main pathogenic bacterium of the cultured orange spotted grouper, which has led to considerable economic losses [6,8].

Currently, vaccines have been developed as a sustainable alternative against *V. alginolyticus*. Some functional or structural proteins of *V. alginolyticus* in recombinant form have been evaluated for vaccine candidates, and attempts were also made to use recombinant *V. alginolyticus* vaccine via intramuscular or intraperitoneal injection to control the bacterial spread [9,10]. DNA vaccine is an effective form in aquaculture, which can express antigenic protein in animal tissues and then induce host immune response [11]. Briefly, plasmid DNA is firstly taken up by host cells following vaccination, and then antigenic proteins are expressed and synthesized using host's cellular machinery, imitating natural infection caused by intracellular pathogen [12]. DNA vaccines offer a way of immunization that overcomes some of the disadvantages of traditional live attenuated, killed or subunit vaccine, which may accompany with risk of infection and high costs. They can also induce strong and long-lasting humoral and cellular immunity, which have made them attractive in vaccine development [13]. In

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aquaculture, DNA vaccines have been widely studied and most of them have already been proved to be effective against pathogens, such as infectious haematopoietic necrosis virus [14], infectious pancreatic necrosis virus [15], hirame rhabdovirus [16], *Aeromonas veronii* [17], *V. anguillarum* [18] and *Streptococcus iniae* [19].

Scientists are now focused on improving and increasing the potency of DNA vaccines, which include the improvements of vectors and the co-vaccination of molecular adjuvants such as cytokines with the antigen to enhance the immune responses. Several studies reported that the use of cytokines as vaccine adjuvants to augment the initial host responsiveness has been widely explored in mammals, and many cytokine genes like IL-1β and TNFα have been identified in many fish species and many details concerning their immunological role are also demonstrated [20,21]. Myeloid differentiation factor 88 (MyD88) is an adapter molecule to activate the signaling cascades and produces inflammatory mediators involved in the interleukin-1 receptor (IL-1R) and Toll-like receptor (TLR)-mediated activation of nuclear factor-kappaB (NF-κB) [22]. A study showed MyD88 could be as vaccine adjuvants in DNA vaccine to enhance vaccine immunogenicity [23]. The antigenic peptide, AcfA, an accessory colonization factor A protein of *V. alginolyticus*, had been reported as an efficient antigenic protein in our previous study [9]. In this study, we explore an optimized DNA vaccine co-encoding AcfA peptide with MyD88 adjuvant, and the immune-protective efficiency of co-injection of the adjuvant pcMyD88 with DNA vaccine pcacfA and the immune response induced by the vaccine were investigated in orange spotted grouper.

2. Materials and methods

2.1. Fish and bacteria

Healthy orange spotted grouper weighing approximately 50 g were obtained from a local fish farm (Donghai island, Zhanjiang, China) and reared in 1000 l tanks for two weeks before experimental manipulation. All fish were fed twice daily with commercial feed and were acclimatized at 24–26 °C. Fish were anaesthetized with tricaine methanesulfonate (MS222) (Sigma, Beijing, China) prior to injections and blood collection. Animal experiments were carried out in strict accordance with the regulations of local government and approved by the Ethics Committee of Guangdong Provincial Key Laboratory of Pathogenic Biology and Epidemiology for Aquatic Economic Animals.

V. alginolyticus strain HN1021 was isolated from diseased orange spotted grouper and maintained in our laboratory. *V. alginolyticus* was grown in tryptic soy broth (TSB, Huankai, Guangzhou, China) supplemented with 2% NaCl at 25 °C, while *Escherichia coli* strains were cultured in Luria broth (LB, Huankai, Guangzhou, China) at 37 °C. When required, the appropriate antibiotics were added: ampicillin (Amp, 100 μg/ml) or chloramphenicol (Cm, 30 μg/ml).

2.2. Preparation of vaccine and adjuvant

Genomic DNA of *V. alginolyticus* strain HN1021 was extracted using the MiniBEST Bacterial Genomic DNA Extraction Kit (Takara, Dalian, China). The primers (Table 1) AcfAF/AcfAR containing *Hin*d III and *Eco*R I cutting sites, respectively, were designed using the *acf*A gene (Genbank accession no. JN172935) to clone *acf*A gene coding mature peptide. The gene *acf*A was amplified according to our previous study [9]. The amplification conditions were performed as following: 94 °C for 5 min, 30 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, followed by 72 °C for 10 min. The PCR product was ligated with pMD-19T vector (Takara), and transformed into competent *E. coli* DH5α cells and sequenced by Shanghai Sangon Biologic Engineering & Technology and Service Co. Ltd. The *acf*A gene was excised from pMD19T-*acf*A by digestion with *Hin*d III and *Eco*R I restriction enzyme (Takara) and inserted into the pcDNA3.1(+) (Invitrogen, Carlsbad, CA, USA) to generate pcacfA. A recombinant plasmid pcacfA containing the *acf*A open reading frame was transformed into *E. coli* DH5α cells and sequenced. The positive clone was grown in LB broth with ampicillin and incubated at 37 °C overnight with shaking. Recombinant plasmid DNA was isolated with MiniBEST Plasmid Purification Kit (Takara) following the manufacturer's instructions and the concentration was measured by the NanoDrop 2000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE) and conserved at −20 °C.

Table 1
Sequences of primers used in this study.

Name	Sequence (5' - 3')	Targeting sequence	Product size (bp)
Constructing plasmids			
AcfAF	CCAAGCTTGGTAATATGGACAAAACAC (*Hin*d III)	*acf*A of *V. alginolyticus*	675
AcfAR	CGGAATTCCGATATTAGAAGTAATAA (*Eco*R I)		
MyD88F	CCAAGCTTGGCTCTCCAACATGGCG (*Hin*d III)	*Myd*88 of *E. coioides*	908
MyD88R	CGGAATTCCGGTCCTTGGTTACGG (*Eco*R I)		
RT-PCR detection			
VfAcfAF	TCAGTTTCGGTATCTGCGGC	*acf*A + pcDNA fragment	726
pcDNAR1	AGGGGCAAACAACAGATGGC		
VfMyD88F	GTGTAAACCCCACCAAAGCG	*Myd*88 + pcDNA fragment	805
pcDNAR2	GGGGCAAACAACAGATGGCT		
Quantitative real-time PCR analysis			
MHCIF	GCCGCCACGCTACAGGTTTCTA	MHCIα	113
MHCIR	TCCATCGTGGTTGGGGATGATC		
MHCIIF	GGAGCCTCAGCCCAGCTTCA	MHCIIα	131
MHCIIR	CCAGTGGGAGGTCCTTCATG		
CD4F	TTTCTGGCCAGCAGCTCAAC	CD4	147
CD4R	CTTCCGGGATGGTGAGATG		
CD8F	CACCACGCCGTGTGTTTGTAG	CD8α	139
CD8R	GTAGACGGTGGTGGCGATGAG		
ILF	TCTGGGCATCAAGGGCACACA	IL-β	122
ILR	CCATGTCGCTGTTCGGATCGA		
TNFF	GCCACAGGATCTGGCGCTACTC	TNF-α	106
TNFR	CTTCCGTCGCTGTCCTCATGTG		
actinF	GGACAGCTACGTTGGTGATGA	β-actin	83
actinR	TGGTCACAATACCGTGCTCAATG		