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Effect of a new recombinant *Aeromonas hydrophila* vaccine on the grass carp intestinal microbiota and correlations with immunological responses

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ABSTRACT

Intestinal microbiota has become an integral component of the fish, and plays a key role in host metabolism, immunity and health maintenance. However, information on the immune responses after vaccine administration in relation to the intestinal microbiota is absent in fish. The present study focused on the effect of a new recombinant *Aeromonas hydrophila* vaccine (Aera) by using a novel functionalized, single-walled carbon nanotubes (SWCNTs) as a delivery vehicle on the intestinal microbiota of grass carp (*Ctenopharyngodon idella*) through the bath immunization, and further explored the immunological responses in intestine, kidney and spleen. By performing deep sequencing, a total of 81,979 valid reads and 609 OTUs obtained from 4 intestine samples were analyzed. We detected 141 genuses, most of which belonged to Firmicutes, Fusobacteria and Proteobacteria. Of note, the quantity of *Aeromonas* in library Aera (after 6 h Aera vaccine pretreatment, fish were transferred to tanks without SWCNTs-Aera for 28 d) and Aera-GD (6 h Aera vaccine pretreated prior to the group injected by *A. hydrophila*) was declined 6.5% and 14.6% compared with the control, respectively. Moreover, the expression of seven immune-related genes (IFN-I, TNF-α, CRP, IL-8, IgM, MHC I and CD8α) in the intestine, kidney and spleen of Aera treated fish was significantly enhanced, which indicated that a better tissue immune response in grass carp was induced by the SWCNTs-Aera vaccine. Therefore, a new recombinant SWCNT-Aera vaccine may represent potentially efficient and immunological role in grass carp intestine to resist *A. hydrophila* infection.

1. Introduction

As a gram-negative bacterium, *Aeromonas hydrophila*, as a major pathogen in almost all animal taxa, produces intestinal inflammation in fish and induces high mortalities in a wide range of fish species at different stages of growth [1–3]. Currently, the bacterium is regarded as one of the major problems in carp aquaculture, causing considerable economic losses in aquaculture industry worldwide [4–6]. Several studies in fish have found that *A. hydrophila* enters the fish body via the gills and damages skin [7], and results in the symptoms that include swelling of tissue, dropsy, red sores, necrosis, ulceration, and hemorrhagic septicemia [8]. Since 2009, *A. hydrophila* infections in aquaculture operations have tremendous economic impact on catfish aquaculture in the southeastern United States and cultured grass carp in China [9,10]. Therefore, many efforts are being directed towards finding effectively preventive measures which may damper the events behind the pathogenic process of *A. hydrophila*. In an attempt to control the spread of this disease, *A. hydrophila* was controlled by fish farmers using wide range of antibiotics in the past. However, antibiotic treatment may be detrimental to the environment and human health [11]. As described previously, a recombinant vaccine via intramuscular injection in an attempt to control the spread of this disease is being developed against *A. hydrophila* but it is currently not commercially available in China [12–14]. Recently, we found that a novel functionalized, single-walled carbon nanotubes (SWCNTs) as a vector for the recombinant protein Aera (a virulence factor that has hemolytic and cytolytic properties) through bath immunization could produce specific antibodies in grass carp to resist *A. hydrophila* infection [3].

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It is well known that intestinal microbiota may also play an important role in fish health [15,16], and commensal colonization brings many physiological, metabolic and immunological benefits to the host. Specifically, the mucosal immune system has evolved to permit the colonization of mucosal surfaces with complex and diverse microbial communities [17,18]. By shared "microbe-associated molecular patterns" (MAMPs), pathogens and commensals are recognized by the pattern recognition receptors (PRRs) of the immune system [19,20]. When homeostasis is broken, commensals could access to the host's internal milieu and damage the host [17]. As a common human enteric pathogen, *A. hydrophila* attracted the public concerns since 1970s, because it could cause the acute diarrheal disease. Meanwhile, it was found that *A. hydrophila* also composed a part of the intestinal microflora of healthy fish [21], and was considered an opportunistic agent that did not cause problems in healthy fish [22]. So far, several investigators have demonstrated that orally administered *A. hydrophila* can result in a systemic infection in fish [23,24] and that the intestinal tract is a site of adhesion. Therefore, commensal colonization of *A. hydrophila* in intestine requires precise homeostatic regulatory mechanisms from the host's immune system. However, a particular microbial community composition associated with an anti-*A. hydrophila* vaccine is unknown. According to our finding [3], the vaccine Aera can provide an immune protection in grass carp to resist *A. hydrophila* infection, and further it is necessary to identify its role by determining the fish intestinal bacterial community and associated immune response.

At the present, intestinal regulation is one of the hottest topics in biological sciences and most researchers are dealing with the local immune response. Through a 16S rRNA clone library analysis, the present study therefore aims to (1) characterize the intestinal bacterial community of grass carp pretreated with Aera, and (2) reveal the association between intestinal microbiota, especially concerning *A. hydrophila*, and the vaccine Aera. Furthermore, we evaluated the immune response of intestine, kidney, and spleen induced by Aera vaccine after bath administration. This study will help us to further determine the efficient and specific Aera vaccine roles in grass carp.

2. Material and methods

2.1. Experimental fish and bacterial strain

Grass carp (n = 2000) were obtained from the Xinmin Aquatic Breeding Center (Heyang, Shaanxi, China), with the total length and body weight of 3.0 ± 0.5 cm and 1.0 ± 0.1 g (data are presented as mean values ± SD), respectively. Fish were acclimatized in five 300 L aquaria with 28 °C in aerated water under laboratory conditions for two weeks prior to the beginning of experiments and fed to apparent satiation twice a day (10:00, 16:00) with a diet of commercial dry pellets (Wuhan Tianlong Feed Company, Wuhan, China). Based on the primers combination strategy of Vázquez-Juárez et al. [25], grass carp were checked randomly to verify pathogen free status of *A. hydrophila* prior to the trial. All protocols strictly adhered to the guide for care and use of laboratory animals, and were approved by Northwest A&F University animal protection committee.

A. hydrophila strain XS91-4-1 was preserved in our laboratory with 20% glycerol at −80 °C. During the experimental period, *A. hydrophila* were cultured in Luria Bertani (LB) broth at 28 °C and grown overnight with constant shaking.

2.2. Purification of recombinant protein and preparation of vaccine

The expression and purification of the recombinant Aera protein and preparation of SWCNTs-Aera vaccine were performed as described previously [3]. Briefly, the Aera gene was isolated from genomic DNA of *A. hydrophila* by PCR, using two oligo nucleotide primers, cloned into the pET32a expression vector and transformed into *Escherichia coli* for the expression of the N-terminal His-tag fusion proteins [26]. The *E. coli* cells, each containing pET32a-Aera recombinant plasmid, were grown to approximately 0.6–0.8 optical density (OD) at 600 nm at 37 °C in LB medium containing 100 mg/ml of ampicillin. The expression of recombinant Aera protein was induced by 1 mM isopropyl-β-D-thiogalacto-pyranoside for 6 h. The SWCNTs were treated by the mixture of concentrated HNO_3/H_2SO_4 (1:3 v/v) at 70 °C for 12 h under reflux with stirring to produce the oxidized SWCNTs (o-SWCNTs) [27]. The SWCNTs-Aera was prepared by covalently bonding the Aera protein on the o-SWCNTs via the diimide-activated amidation process as previously reported [28].

2.3. Experimental design

In vaccination experiments, healthy grass carp were distributed randomly into control group and bath immunization group (150 fish per group). For bath immunization, fish were immersed in 20 mg L^{-1} SWCNTs-Aera and the control group was immersed in PBS for 6 h. At the end of the vaccination, grass carp were transferred to different tanks without SWCNTs-Aera for 28 d. After 28 d, 15 fish in each replicate were euthanized in the laboratory through washrag soaked with 20.0 g/m^3 MS-222. Sampled fish were dissected immediately with sterile scissors. The intestine, kidney and spleen were aseptically removed from their abdominal cavity, separately, where five fish were sampled for analyzing expression of seven immune-related genes through quantitative real-time PCR (qRT-PCR) and the experiment was performed in triplicate replicates. Thereafter, the intestine of three fish (15 fish per replicate) were also collected and pooled together as described elsewhere to extract microbial DNA for deep sequencing [29]. After 2 d, the remaining fish (15 grass carp in each replicate) were intramuscularly injected with 6.5×10^4 cfu mL^{-1} *A. hydrophila*, kept for 10 days and thereafter euthanized for sampling. Subsequently, the intestine was carefully excised and maintained as above, which was also for extracting microbial DNA for deep sequencing. All the samples were placed into sterile polypropylene centrifuge tubes and stored in −80 °C until DNA extraction. All experiments were performed in triplicate.

2.4. DNA extraction and PCR amplification

Microbial DNA was extracted from grass carp intestine samples by using the Bacterial DNA Kit (50) (Omega, USA) following manufacturer's instruction. For each sample, DNA was extracted in triplicate to avoid bias, and the extracts from the same sample were pooled [30]. Purity of DNA extracted was verified by electrophoresis on ethidium bromide staining 1% agarose gels, and concentration was analyzed spectrophotometrically using the M200pro (TECAN, Switzerland). The extracted DNA was stored at −80 °C until use. The V4–V5 region of the bacteria 16S ribosomal RNA gene were amplified by PCR (95 °C for 2 min, followed by 25 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s and a final extension at 72 °C for 5 min). The primers used were 515F 5′-barcode-GTGCCAGCMGCCGCGG-3′ and 907R 5′-CCGTCAATTCMTTTRAGTTT-3′, where barcode is an eight-base sequence unique to each sample. PCR reactions were performed in triplicate 20 μL mixture containing 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase, and 10 ng of template DNA.

2.5. Illumina MiSeq sequencing

Deep sequencing was performed on Illumina sequencing with MiSeq using paired-end technology provided by Shanghai Majorbio

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