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Delivery of DNA vaccine using chitosan–tripolyphosphate (CS/TPP) nanoparticles in Asian sea bass, *Lates calcarifer* (Bloch, 1790) for protection against nodavirus infection

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ABSTRACT

The present study examines the efficacy of DNA vaccine against nodavirus through oral route using CS/TPP (chitosan–tripolyphosphate) nanoparticles encapsulation. The RNA2 capsid protein gene of nodavirus was used to construct DNA vaccine using pcDNA 3.1, a eukaryotic expression vector and the construct was named as pFNCPE. The size of the RNA2 capsid protein gene was 42 kDa and it was named as pFNCPE42. The CS/TPP nanoparticles were used to deliver the constructed plasmid. *In vitro* and *in vivo* expressions of FNCP (fish nodavirus capsid protein) gene were observed in sea bass kidney cell line (SISK) and in fish, respectively by fluorescent microscopy. The cytotoxicity of CS/TPP–pFNCPE42 DNA nanoparticles was evaluated by MTT assay using fish cell line. Distribution of DNA vaccines in different tissues was studied in fish fed with the pFNCPE42-DNA encapsulated in CS/TPP nanoparticles and the expression of the gene was confirmed by PCR, RT-PCR, immunohistochemistry and ELISA analysis. The results indicate that DNA vaccine can be easily delivered into fish by feeding with CS/TPP nanoparticles. After oral vaccination Asian sea bass were challenged with nodavirus by intramuscular injection. A relative percent survival (RPS) rate of 60.0% was recorded. This study suggested that CS/TPP nanoparticles were promising carriers for plasmid DNA vaccine and might be used to vaccinate fish by oral approach.

1. Introduction

Fish nodavirus the causative agent of VNN, is a small, spherical, non-enveloped virus with a genome consisting of two single stranded RNA segments: RNA1 (3.1 kb), which encodes the RNA-dependent RNA polymerase responsible for the replication of the genome, and RNA 2 (1.4 kb), which encodes the capsid protein and RNA 3(0.4 kb) (Iwamoto et al., 2005a,b; Nishizawa et al., 1995; Sommerset and Nerland, 2004). The main target organ for nodavirus in fish is the central nervous system (CNS), including the brain, spinal cord and retina, where it causes extensive cellular vacuolation and neuronal degeneration (Mori et al., 1992).

Vaccine is a biologically prepared antigen which helps to improve the immunity in animals against a particular disease or a group of diseases. Different types of vaccines such as heat or formalin killed whole cell vaccine, recombinant protein vaccine and DNA vaccines have been tried to protect the fish nodavirus (Pakingking et al., 2010; Sideris, 1997; Sommerset et al., 2003, 2005; Vimal et al., 2014; Yuasa et al., 2002). Previous reports indicate that development of DNA vaccine using gene encoding glycoprotein of viral hemorrhagic septicemia virus (VHSV) and challenge experiments revealed that the immunity established is cross protective against heterologous fish rhabdoviruses and also against nodavirus (Sommerset et al., 2003). However, DNA vaccines, constructed by cloning the encoding region of RNA2 from AHNV and SJNNV, have not proven efficacious (Sommerset et al., 2001, 2003).

Gene transfer for transient expression in fish is very important for the application of DNA vaccines to prevent viral and bacterial diseases of economically important fish species (Anderson et al., 1996). Chitosan/tripolyphosphate nanoparticles (CS/TPP) have been used as an alternative to chitosan to encapsulate peptides, proteins, pDNA and siRNA by various workers (Calvo et al., 1997; Cuna et al., 2009; Fernandez et al., 1999; Gan and Wang, 2007; Gan et al., 2005; Vila et al., 2004; Vimal et al., 2012, 2013; Wang et al., 2009; Yang et al., 2009). A technique for developing chitosan nanogels by adding a cross-linking agent, *i.e.* tripolyphosphate (TPP), into the aqueous phase containing chitosan has been developed (Calvo et al., 1997). Chitosan-TPP nanoparticles with entrapped siRNA have been found to be better vector siRNA delivery vehicles compared to chitosan siRNA complexes (Katas and Alpar, 2006). An ionic gelation technique for the encapsulation of different nucleic acids (plasmid DNA and short oligonucleotides) into chitosan-TPP nanoparticles has been adapted and

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its potential as gene delivery nanocarrier was evaluated (Csaba et al., 2009; Rajeshkumar et al., 2009). In addition, chitosan-TPP can penetrate deep into tissues through fine capillaries and this allows efficient delivery of proteins, drug and plasmid DNA in the body. In the present study, an attempt was made to make use of chitosan/tripolyphosphate nanoparticles (CS/TPP) as an alternative to chitosan to deliver the DNA vaccine through oral route in Asian sea bass for protection against nodavirus.

2. Materials and methods

2.1. Virus isolate and virus propagation

The fish nodavirus used in the present study was isolated from infected Asian sea bass (*Lates calcarifer*) larvae during a massive outbreak in sea bass hatcheries located in Chennai and Nagapattinam of Tamilnadu, India (Azad et al., 2005; Parameswaran et al., 2008). Nodavirus was propagated using sea bass kidney cell line (SISK) as described by Sarath Babu et al. (2013).

2.2. Collection and maintenance of experimental animals

Healthy juveniles of Asian sea bass (*L. calcarifer*) fish (10 to15 g in body weight) were collected from grow-out ponds of Central Institute of Brackishwater Aquaculture, Chennai and transported in live condition to the laboratory. In the laboratory, the animals were maintained in 500 l tanks containing UV-treated seawater (salinity 30 ppt) with continuous aeration at 24 °C. The fish were fed twice a day with boiled fish meat during the acclimatization and experimental periods. After acclimatization, the fish were placed in separate tanks for experimental purpose.

2.3. Construction and preparation of DNA vaccine

The gene encoding for ORF of the major capsid protein, approximately 1.017 kb in length, was amplified using specific primer set (Table 1) and subsequently cloned into a eukaryotic expression vector pcDNA3.1 (Invitrogen), behind the early cytomegalovirus promoter, yielding pcDNA3.1–FNCPE42. The pcDNA3.1–FNCP42 was verified using *Hind III* and *EcoR I* endonuclease analysis, and the recombinant plasmid was then transformed into *Escherichia coli* DH5α cells. Recombinant clone was selected based on ampicillin resistance and confirmed by DNA sequencing. Plasmid was named as pFNCPE42. Plasmid was purified with the EndoFree Plasmid Mega purification kit (Qiagen) according to the manufacturer's instructions, aliquoted at 1 μg/ml in sterile endotoxin free phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4), and stored at −20 °C until further use.

2.4. Preparation of CS/TPP nanoparticles

The synthesis and characterization of CS/TPP nanoparticles have been described in our earlier publication (Vimal et al., 2012).

Table 1
Cloning primers used for amplification of capsid protein gene for eukaryotic expression vector.

Primer	Product size	Sequence (5′-3′)	Annealing temp.
FNCPE 42-F	1017 bp	CGCAAGCTTACCATGGTACGCAAAGGTGAGAAG *Hind III*	55 °C
FNCPE 42-R		CCCGAATTCCCGGTTAGTTTTCCGAGTCAA *EcoR I*	

Sequences were taken from GenBank accession no. KF146304.

2.5. Preparation of CS/TPP–DNA (pFNCPE42) nanoparticles

The solutions of CS/TPP nanoparticles (pH 5.5) and pFNCPE42 DNA (100 μg/ml) were preheated to 55 °C separately. An equal volume of heated CS/TPP and pFNCPE42 DNA solutions was quickly mixed together and vortexed (2500 rpm) for 30 s following the protocol described by Bozkir and Saka (2004). The pFNCPE42 DNA-loaded CS/TPP particles were carefully transferred to centrifuge tubes and nanoparticles were separated by centrifugation at 20,000 rpm for 30 min at 10 °C. The supernatant was collected and the unbound pFNCPE42 DNA content in the supernatant was quantified by UV spectrophotometer at 260 nm. The encapsulation efficiency (EE) was calculated using the following equation as described in our earlier publication (Vimal et al., 2012).

2.6. In vitro transfection of pFNCPE42 in sea bass kidney cell line (SISK)

The SISK cells (Sahul hameed et al., 2006) were grown on coverslips (22 × 22 mm) for 24 h, after subconfluent monolayer cells were transfected with CS/TPP–pFNCPE42 DNA nanoparticles. The amount of CS/TPP–DNA nanoparticles (CS/TPP–pFNCPE42) equivalent to 2–4 μg DNA of pFNCPE42 was added and incubated with the cells for 8 h followed by 24 h incubation in fresh complete medium (Leibovitz's L-15) In control experiments, cells received the same amount of DNA (pFNCPE42 in sodium sulfate buffer). All transfection experiments were performed in triplicate. After 48 h, the cells were fixed with 3.7% p-formaldehyde for 10 min at 4 °C, washed with PBS, permeabilized with 0.1% Triton X-100 at 4 °C for 4 min, and then blocked in PBS containing 1% bovine serum albumin (BSA) for 30 min at room temperature. Polyclonal antibody raised in rabbit against capsid protein of nodavirus was diluted (1:50) in PBS with 1% BSA and directly added to the fixed cells and kept for 2 h at room temperature. Then the cells were washed with wash buffer, followed by addition of the goat anti-rabbit IgG secondary antibody (IgG) conjugated with FITC at a dilution of 1:50 for 45 min at room temperature. The cells were washed and mounted with antifade 1, 4-diazobicyclo-2, 2, 2-octanex (DABCO) in mounting medium (Sigma). The cover slips were observed under a fluorescence microscope (Carl Zeiss, Germany).

2.7. In vitro cytotoxicity of CS/TPP–pFNCPE42 DNA nanoparticles studies by the MTT assay

The cytotoxicity of CS/TPP–pFNCPE42 DNA nanoparticles was evaluated using kidney cell line of sea bass (SISK) following the protocol described by Vimal et al. (2012).

2.8. Preparation of fish feeds

The fish feed was made from a mixture of sifted flour, mashed fry (commercially available fish meal) and distilled water at the ratio of 10:1:5 (w/w/v). These ingredients were mixed until a very soft biscuit-textured dough was obtained. Each of the lyophilized pFNCPE42 DNA–CS/TPP complex preparations was added to 1 g (dry weight) fish flake dough and was spread thinly (approximately 1.5 mm) on a cupcake-sized mold of a no-stick baking pan. The dough was heated at 35 °C for 45 min in a drying oven and flaked by crumbling (Ramos et al., 2005).

2.9. Vaccination and challenge

Juveniles of Asian sea bass (10–15 g body weight) were used in challenging experiment to evaluate the efficacy of DNA vaccine delivered by CS/TPP nanoparticles against nodavirus. Prior to vaccination, the fish were acclimatized for 1 week in the laboratory. Sea bass were divided into four groups (120 per group) for challenging experiment. In Group I, the fish were fed with fish flakes containing CS/TPP–PBS. In Group II, the fish were fed with fish flakes containing the complex of