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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

CS/TPP–pcDNA3.1. In Group III, the fish were fed with fish flakes containing CS/TPP–pFNCPE42 DNA of nodavirus at the concentration 100 μg/ml plasmid complex per fish. In Group IV, the fish were fed with fish flakes containing naked pFNCPE42 DNA. In all the groups, the fish were fed with experimental feed for one day at the rate of 3% body weight for three times. After experimental feed, the fish were fed with normal pellet feed at the rate of 3% body weight, 3 times a day. After 3rd week post-vaccination, the fish in all the groups were challenged with an intramuscular injection of nodavirus at a dose of $1 \times 10^{6.5}$ $TCID_{50}$ $fish^{-1}$. After 30 days of post-challenging, the fish were examined daily and cumulative percentage mortality was recorded. Relative percent survival (RPS) was calculated using the following formula: (1 − % mortality of vaccinated fish / % mortality of control fish) × 100.

In another experiment, fish were divided into four groups (60 per group) as mentioned above. After treatment as described above in different groups, different tissue samples namely heart, intestine, gill, muscle and liver tissue were collected from 40 fish at 10 days post-vaccination for PCR, RT-PCR, ELISA and immunohistochemistry analyses. At 3rd week post-vaccination, blood sample was collected from the remaining fish to determine the antibody response in vaccinated fish by ELISA as described by Rajesh kumar et al. (2007). The experiment was carried out in triplicates.

2.10. Tissue distribution of pFNCPE42 by PCR

The tissue distribution of pFNCPE42 in fish fed with feed containing CS/TPP–pFNCPE42 was confirmed by PCR. The DNA was extracted from the tissue samples (heart, intestine, gill, muscle and liver) following the protocol of Vimal et al. (2012).

2.11. Transcriptional analysis of pFNCPE42 gene by RT-PCR

To confirm the transcriptional analysis the samples were subjected to RT-PCR. Total RNA was extracted from the (heart, intestine, gill, muscle and liver) tissues using Trizol reagent (Invitrogen, Carlsbad, USA), as per manufacturer's instructions of our earlier protocol (Vimal et al., 2012).

2.12. In vivo expression of FNCP by immunohistochemistry

The expression of FNCP gene in different tissues of fish was analyzed by immunohistochemistry. The samples (heart, intestine, gill, muscle and liver) were collected from the fish fed with feed containing CS/TPP–pFNCPE42 and fixed with 10% formaldehyde (made in 0.1 M PBS, pH 7.4). After 48 h, the tissue samples were processed and embedded in paraffin wax, and then cut into sections of 5 μm thickness using microtome. The paraffin sections were deparaffinized in xylene, and hydrated through descending graded levels of alcohol to distilled water. The sections were then treated with trypsin (0.1% w/v in PBS, biochemical grade, Hi Media, India) for 10 min and washed twice with PBS-Tween 20 (T-20, Hi Media, India; 0.01% v/v with PBS). Non-specific antibody binding sites were blocked for 30 min using 3% bovine serum albumin (in PBS, pH 7.4) and washed with PBS/T. The sections were treated with the polyclonal antibody rabbit antiserum specifically raised against capsid protein of nodavirus at a dilution of 1:50 (sterile PBS, pH 7.4) for 1 h in a humid chamber. Sections were washed with PBS-T and treated with goat anti-rabbit FITC conjugate at a dilution of 1:50 for 30 min at room temperature. After washing, it was mounted using glycerol following the protocol of Rajesh kumar et al. (2007). The slides were observed under a fluorescent microscope (Carl Zeiss, Germany).

2.13. Enzyme-linked immunosorbent assay (ELISA)

The expression of FNCP gene in different tissues of fish was also confirmed by ELISA using the antiserum raised against capsid protein of nodavirus. The wells of flat-bottomed ELISA plate were coated with the suspension of tissue samples of fish fed with CS/TPP–pFNCPE42 in PBS overnight at 4 °C. The plates were then washed thoroughly with PBS and blocked with 2% BSA in PBS for 1 h at 37 °C. Subsequently, the plates were washed thoroughly with PBS/T and incubated with antiserum raised against capsid protein at 37 °C for 2 h. The plates were washed with PBS/T and PBS three times each for 2 min and further incubated with 100 μl of goat anti-rabbit IgG conjugated with alkaline phosphatase for 1 h. The plates were washed with PBS/T and PBS three times each for 2 min and developed with the substrate p-nitrophenyl phosphate in substrate buffer. The optical density was measured at 405 nm using an automated ELISA reader (Labsystems, USA).

2.14. Analysis of antibody response

After 3rd week post-vaccination, fish from each treatment group were assayed for antibody response against FNCPE42 by sandwich enzyme-linked immunosorbent assay (ELISA) following the protocol described previously (Rajesh Kumar et al., 2007).

2.15. Statistical analysis

Data are expressed as mean ± SD. Statistical analysis was performed using one-way ANOVA for antibody response, and a log rank test using the Kaplane Meier method was used for survival analysis. $p < 0.05$ was taken to indicate statistical significance.

3. Results

3.1. Cloning of nodavirus viral capsid protein

The major nodavirus coat protein is a viral capsid protein which was cloned into eukaryotic expression vector and used as antigen for our nodavirus oral DNA vaccine.

3.2. Encapsulation efficiency

The encapsulation efficiency of CS/TPP to encapsulate pFNCPE42 was determined and the results revealed a high encapsulation efficiency of 83.6% of DNA binding with CS/TPP nanoparticles.

3.3. Tissue distribution of DNA by PCR

The distribution of pFNCPE42 DNA in different tissues of fish fed with pFNCPE42–CS/TPP was determined by PCR and the results revealed the presence of pFNCPE42 in the heart, intestine, gills, muscle and liver tissues of fish. The PCR product was observed at the corresponding size of 1017 bp specific to the FNCP gene of nodavirus. The results are shown in Fig. 1. No amplification was observed in fish fed with pellets containing CS/TPP–pcDNA 3.1 or CS/TPP–PBS.

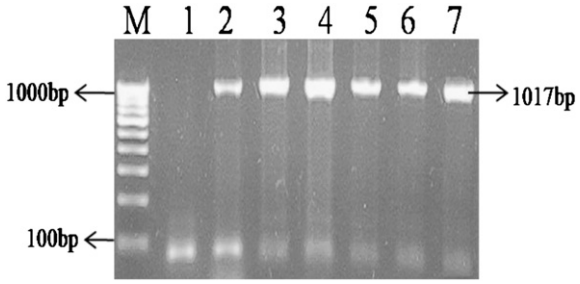

Fig. 1. PCR analysis showing the distribution of pFNCPE42 in different organs of fish fed with CS/TPP–pFNCPE42 DNA. Lane M — Marker, lane 1 — Negative, lane 2 — Heart; lane 3 — Intestine, lane 4 — Gills, lane 5 — Muscle, lane 6 — Liver, and lane 7 — Positive.

3.4. In vitro and in vivo transcription analysis of pFNCPE42 by RT-PCR

Transcription analyses of the CS/TPP encapsulated pFNCPE42 gene in different tissues of vaccinated fish and SISK cell line were performed by a RT-PCR reaction on DNase I-treated RNA from SISK cell line transfected with CS/TPP–pFNCPE42 complex and the empty plasmids (CS/TPP–pcDNA3.1). Amplified product of 1017 bp was obtained using the specific primer set (Table 1) and the results shown in *in vitro* (Fig. 2) and *in vivo* (Fig. 3). No amplification was observed in fish fed with fish flakes containing control pcDNA3.1 and SISK cells transfected with control pcDNA3.1.

3.5. In vitro expression of pFNCPE42 gene

The ability of the constructed CS/TPP–pFNCPE42 DNA vaccine complex to express FNCPE42 in eukaryotic (SISK cell line) cells transfected with pFNCPE42 and in the negative control pcDNA3.1 was studied. After 48 h transfection, only pFNCPE42-transfected cells expressed protein FNCPE42, which was detected by fluorescent microscope and the results are shown in Fig. 4A.

3.6. In vivo expression of pFNCPE42 gene

In vivo expression of FNCPE42 protein could also be detected by immunohistochemistry in different tissues (heart, intestine, gills, muscle and liver tissues) in fish orally vaccinated with CS/TPP–pFNCPE42 complex (Fig. 4B) and the ELISA results are shown in Table 2.

3.7. In vitro cytotoxicity by MTT assay

Cytotoxicity of CS/TPP encapsulated pFNCPE42 was evaluated by the 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Viability of SISK cells incubated with the pFNCPE42–CS/TPP complexes was more than 90%. No significant changes in cell morphology were observed in comparison to control cells. The result showed that the cytotoxicity of pFNCPE42–CS/TPP was quite low.

3.8. Antibody response to DNA immunization

The humoral immune response of Asian sea bass to CS/TPP mediated oral immunization with the pFNCPE42 DNA vaccine was assessed by ELISA at 3rd week after oral vaccination (Fig. 5). Fish orally vaccinated with pFNCPE42 produced very low levels of anti-FNCPE42 antibody. Antibody levels against FNCPE42 were statistically significant at the serum dilution 1:1000 in vaccinated fish in relation to negative control groups of fish treated with PBS buffer and pcDNA3.1 alone.

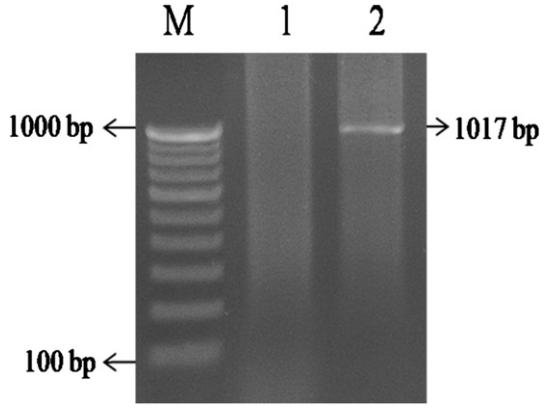

Fig. 2. Reverse transcriptase PCR (RT-PCR) analysis showing the transcription analysis of pFNCPE42 DNA in SISK cell line (*in vitro*). Lane M — Marker, lane 1 — SISK cell line transfected with pcDNA3.1 alone, and lane 2 — SISK cell line transfected with pFNCPE42.

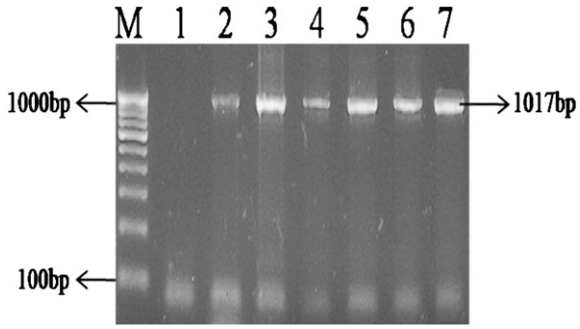

Fig. 3. Reverse transcriptase PCR (RT-PCR) analysis showing transcriptional analysis of capsid protein gene of nodavirus in different organs of fish fed with CS/TPP–pFNCPE42 DNA. Lane M — Marker, lane 1 — Negative, lane 2 — Heart; lane 3 — Intestine, lane 4 — Gills, lane 5 — Muscle, lane 6 — Liver, and lane 7 — Positive.

3.9. Protection of sea bass from nodavirus challenge

Asian sea bass orally immunized with the DNA vaccine encoding for pFNCPE42 was challenged for protection against nodavirus by intramuscular injection. Mortalities following exposure to the virus were lower in fish orally vaccinated with pFNCPE42–CS/TPP compared to those of the naked pFNCPE42 and control groups, which were given PBS buffer and pcDNA3.1 alone. Cumulative percent mortality rate was also calculated (Fig. 6). The orally CS/TPP–pFNCPE42 DNA vaccinated fish were protected from the virus with 60% of RPS which was significant with survival analysis.

4. Discussion

Viral nervous necrosis (VNN) is one of the most important viral diseases in aquaculture. The VNN is a major problem in the farming of marine fish species, causing high mortality in larvae and juveniles (Chua et al., 1995; Munday et al., 1992). The diagnosis and control of nodavirus infection are of great economical importance in commercial fish farming. Because of the economic loss caused by the disease, the aquaculture industry is increasingly relying on vaccines for disease prevention. Several vaccines have been developed by various workers against nodavirus and they are inactivated vaccines (Kai and Chi, 2008), recombinant vaccine (Sideris, 1997; Sommerset et al., 2005) and DNA vaccine (Sommerset et al., 2003). Vaccine preparations based on the recombinant DNA technology have shown promising results in both laboratory and field trials. DNA vaccines using coat protein genes have already been shown to give partial protection against experimental challenge with VHSV and IHNV (Jensen et al., 2009).

The previous studies revealed that CS/TPP nanoparticles could be used for delivery of various biological molecules such as protein (Xu and Du, 2003), SiRNA (Katas and Alpar, 2006) oligonucleotide and plasmid DNA (Csaba et al., 2009) and ShRNA (Wang et al., 2009). In the present study, the CS/TPP nanoparticles were used to deliver FNCP gene in the fish by oral route and the gene was found to be delivered successfully in different parts of the fish as observed by Csaba et al. (2009). These nanoparticles have also been used to deliver drugs (Lam et al., 2006; Shu and Zhu, 2000). This study indicates that gene expression following oral administration of DNA in gene delivery vehicles is possible in fishes. The complex coacervation method was followed to prepare DNA–CS/TPP and our results indicate that this method yielded a high encapsulation efficiency of 81.6%. Our results suggest that ionically cross-linked CS/TPP nanoparticles would be suitable for encapsulation of DNA molecule. A similar result was reported for cross-linked CS/TPP–plasmid and CS/TPP–oligonucleotide complexes (Csaba et al., 2009). Cytotoxicity of pFNCPE42–CS/TPP nanoparticles was evaluated by the 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyltetrazolium bromide (MTT) assay using sea bass kidney cell line (Lleres et al., 2001). The

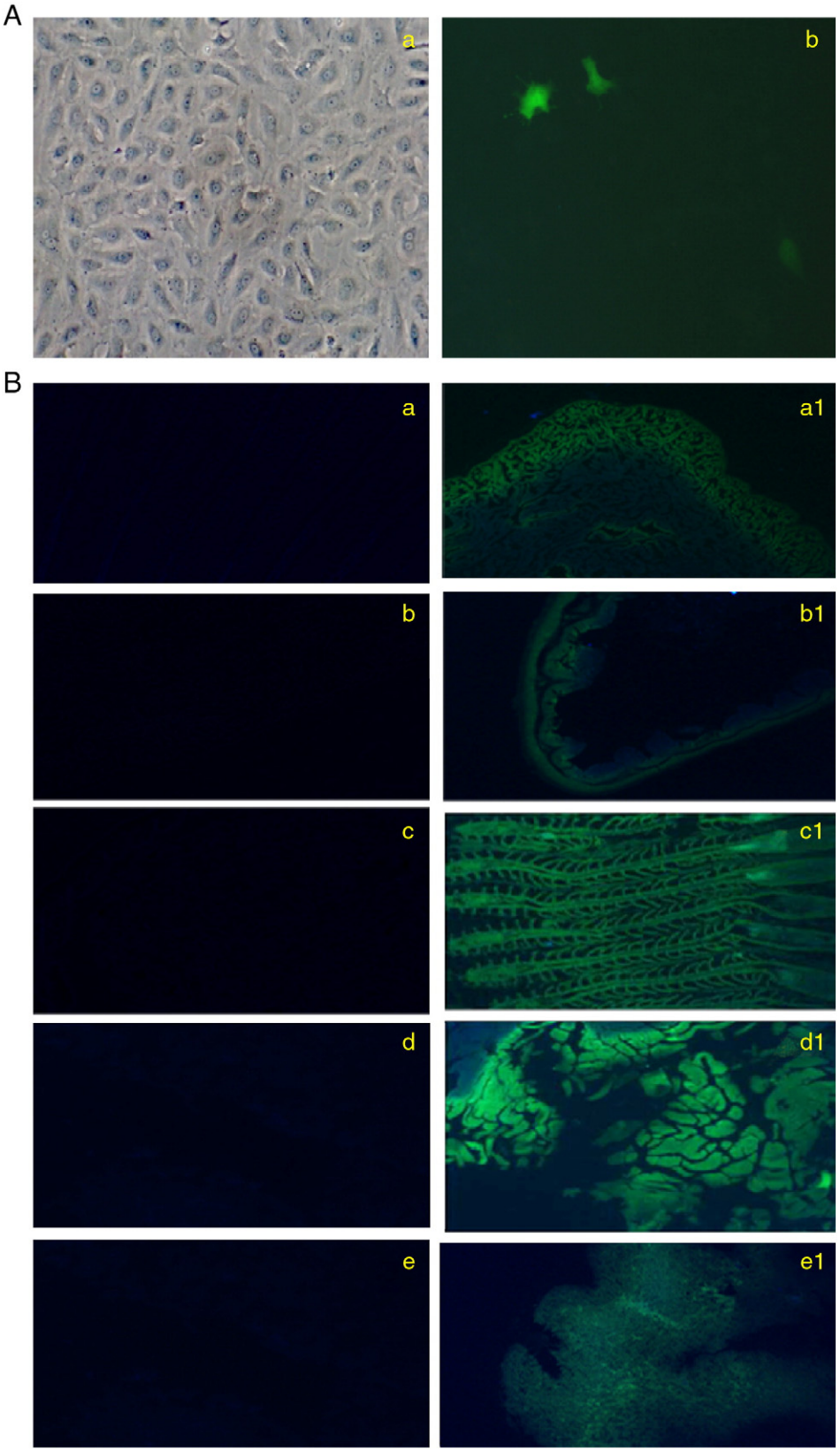

Fig. 4. A. *In vitro* gene expression of CS/TPP nanoparticle encapsulated pFNCPE42 DNA in sea bass kidney cell line by immunofluorescence detection. (a) pcDNA 3.1–CS/TPP transfected sea bass kidney cell line, (b) pFNCPE42–CS/TPP transfected sea bass kidney cell line. B. *In vivo* gene expression of CS/TPP nanoparticles encapsulated pFNCPE42 DNA by immunofluorescence detection in orally vaccinated Asian sea bass in different tissues sample. a to e — Asian sea bass orally vaccinated with CS/TPP–pcDNA3. (a) Heart tissue, (b) intestine tissue, (c) gill tissue, (d) muscle tissue, and (e) liver tissue. a1 to e1 — Asian sea bass orally vaccinated with CS/TPP–pFNCPE42 DNA. (a1) Heart tissue, (b1) intestine tissue, (c1) gill tissue, (d1) muscle tissue, and (e1) liver tissue.

Table 2
In vivo gene expression of FNCP gene of nodavirus in different organs of fish fed with CS/TPP–pFNCPE42 DNA by ELISA.

Organs analyzed	Mean ELISA OD (405) ± SE			
	CS/TPP–PBS	CS/TPP–pcDNA3.1	CS/TPP–pFNCPE42	Naked pFNCPE42 DNA
Heart	0.029 ± 0.003	0.016 ± 0.002	0.929 ± 0.018	0.334 ± 0.028
Intestine	0.038 ± 0.003	0.027 ± 0.002	0.786 ± 0.029	0.281 ± 0.046
Gills	0.047 ± 0.004	0.038 ± 0.003	0.866 ± 0.029	0.301 ± 0.038
Muscle	0.062 ± 0.004	0.043 ± 0.004	1.067 ± 0.026	0.454 ± 0.026
Liver	0.079 ± 0.006	0.051 ± 0.004	0.639 ± 0.033	0.249 ± 0.056

results showed that pFNCPE42–CS/TPP nanoparticles were poorly cytotoxic and caused less than 10% cell death. It has been reported that CS/TPP nanoparticles were less toxic than other cationic polymers such as poly-L-lysine and polyethyleneimine *in vivo* and *in vitro* (Capan et al., 1999; Csaba et al., 2009; Katas and Alpar, 2006).

Tissue distribution of pFNCPE42 was studied in fish fed with CS/TPP–pFNCPE42 in the heart, intestine, gill, muscle and liver by PCR and the results revealed the presence of plasmid in all above said organs. In addition, RT-PCR was also carried out to study the expression of FNCP and the results revealed the expression of pFNCPE42 in different tissues after oral vaccination with CS/TPP nanoparticles loaded with pFNCPE42. Previous studies showed similar results with CS/TPP nanoparticles and chitosan microspheres (Rajesh Kumar et al., 2008; Tian et al., 2008; Vimal et al., 2012). The expression of FNCP in different tissues of fish fed with CS/TPP–pFNCPE42 was also further confirmed by immunohistochemistry and ELISA analyses. The results of *in vitro* and *in vivo* gene expression of pFNCPE42 gene have been carried out to investigate the potential of CS/TPP nanoparticles to transfer exogenous gene into eukaryotic cells. The expression of the gene was observed in the organ (heart, intestine, gills, muscle and liver) tissues of fish fed with feed containing pFNCPE42–CS/TPP nanoparticles. The result of the present study showed that oral vaccination of Asian sea bass with CS/TPP nanoparticles encapsulated pFNCPE42-DNA vaccine induced moderate protection against experimental challenge with nodavirus, which indicated that this method was safe and effective.

In conclusion, our results showed that CS/TPP nanoparticles loaded with pFNCPE42-DNA vaccine conferred significant protection to Asian sea bass against nodavirus after oral administration. Therefore, CS/TPP may represent a potentially safe and efficient oral vaccine carrier against bacterial and viral pathogens of fish. CS/TPP nanoparticle is a suitable material for efficient non-viral gene and DNA vaccines delivery in aquaculture systems.

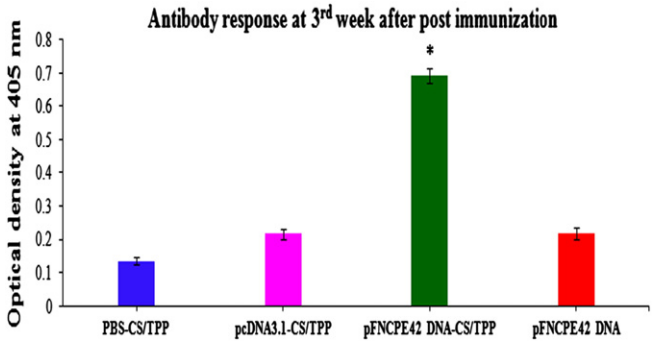

Fig. 5. Detection of anti-FNCPE antibody in serum collected from fish orally immunized with plasmid DNA vaccine (CS/TPP–pFNCPE42) and naked pFNCPE42 and control fish immunized with PBS and plasmid control (pcDNA3.1). Each column represents the mean optical density (OD) among replica groups measuring data at a serum dilution of 1:1000. Statistical analysis was performed using one-way ANOVA for antibody response between treatment and control groups. * Denotes significant differences of pFNCPE42 DNA–CS/TPP ($p < 0.05$).

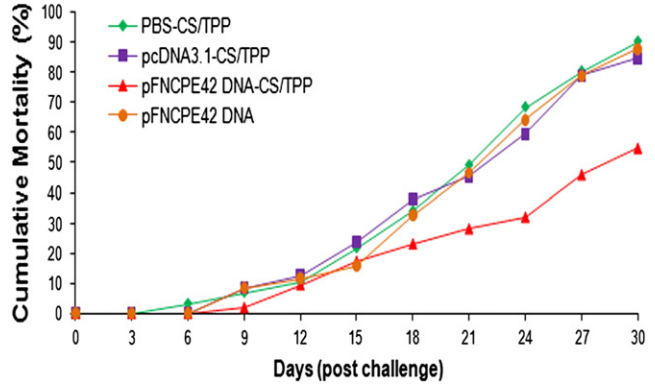

Fig. 6. Cumulative percent mortality in CS/TPP–pFNCPE42 DNA vaccinated and control sea bass after challenge with nodavirus at different time intervals.

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