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Poly $_D$, L-lactide-co-glycolic acid (PLGA)-encapsulated CpG-oligonucleotide (ODN) on immune response in *Cyprinus carpio* against *Aeromonas hydrophila*

Govintharaj Yogeshwari[1], Chandrasekar Jagruthi[1], Ramasamy Harikrishnan[2,*]

[1]Department of Biotechnology, Bharath College of Science and Management, Thanjavur-613 005, Tamil Nadu, (INDIA)

[2]Department of Zoology, Pachaiyappa's College for Men, Kanchipuram - 631 501, Tamil Nadu, (INDIA)

E-mail: rhari123@yahoo.com

ABSTRACT

The effect of Poly $_D$, L-lactide-co-glycolic acid (PLGA) encapsulated ODN 1668 nanospheres (NS) on innate and adaptive immune response in common carp, *Cyprinus carpio* against *Aeromonas hydrophila* is reported. PLGA-encapsulated ODN biodegradable NS did not have a significant impact on particle size as expected of the particles was clearly positive for non-coated and coated PLGA/ODN NS. The myeloperoxidase (MPO) activity and serum haemolysin titre significantly increased in fish injected with ODN 1668 and PLGA-encapsulated ODN 1668 groups on week 4 whereas the respiratory burst (RB) activity significantly increased when administered with ODN 1668 and PLGA-encapsulated ODN 1668 (PLGA-ODN 1668) from weeks 1 to 4. The haemoagglutinating titre was significantly enhanced in PLGA-encapsulated ODN 1668 group on week 2 while in ODN 1668 and PLGA-encapsulated ODN 1668 groups the increase manifested on week 4. The bacterial agglutination titre significantly increased in ODN 1668 and PLGA-encapsulated ODN 1668 groups on weeks 2 and 4. The cumulative mortality in ODN 1668 and PLGA-encapsulated ODN 1668 groups were 10% each whereas it was 15% in PLGA group. This study indicates that single administration of PLGA-encapsulated ODN 1668 nanospheres elicits better immune response than PLGA or ODN 1668 alone in *C. carpio* against *A. hydrophila*.

KEYWORDS

Aeromonas hydrophila;
CpG-oligonucleotide (ODN);
Cyprinus carpio;
Immune response;
Poly $_D$,
L-lactide-co-glycolic acid (PLGA).

INTRODUCTION

In many countries in the world, intensive fish farming has become a key industry in recent decades. *Cyprinus carpio* is the third most frequently introduced species world-wide[1] which grow up to 120 cm in length and around 60 kg weigh[2,3]. For human consumption 3000 years ago of this species and in 1997 produced more than 2,50,000 tonnes in China[4,5]. Carp constitute an ideal candidate species for intensive aquaculture in many Asian countries because of their desirable taste, rapid growth,

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and insatiable demand. It was a greater tolerance of low oxygen levels, pollutants, turbidity, and high salinities than most native fish[6].

With the increasing scale of aquaculture, fishes are reared at high crowded density when environmental conditions are favorable and the fish are healthy, mass mortality will occur if infectious agents are introduced into the farms, causing great financial losses. *Aeromonas hydrophila* is a Gram-negative rod shaped bacterium widely distributed in aquatic environments[7] which the causative agent of motile aeromonad septicemia (MAS), fin-tail rot, and epizootic ulcerative syndrome (EUS) in a wide variety of freshwater fish species[8-12] in Asian countries including China, Philippines, Thailand, and India[13]. *A. hydrophila* is a complex of major disease problem in commercial carp farming and the farmers at present widely used to controlling of MAS using antimicrobial drugs due to unavailable of commercial vaccines such an economically important disease consideration. However, intensive fish farming antimicrobial drugs use for their treatment which associated with increased antibiotic resistance bacteria[14-17]. Although several studies have proved that various vaccine formulations may provide protection[18-22] but it was strain specific and the conventional vaccines are often considered ineffective due to lack of appropriate adjuvant and/or suitable vaccine carrier.

In contrast to other carriers, nano and microparticles are more stable and could elicit both humoral as well as cellular immunity in mammals[23]. By being efficient antigen delivery vehicles they have the potential advantages of reducing the number of injections, enhancing the immune response, and minimize the total antigen dose needed to achieve protection[24-27]. Among the two classes of carriers the Poly $_{D}$, L-lactide-co-glycolic acid (PLGA) nanoparticles have been widely used for controlled delivery of peptides[27], vaccine[28], native and synthetic proteins[29], and nucleic acids[30]; indeed because of their excellent tissue compatibility, biodegradability, non-toxic nature application of nanoparticles have been approved by the Food and Drug Administration for safe use in human and animals[25].

In fish the intra-peritoneal administration of PLGA nanoparticles is reported to stimulate certain non-specific immune response and pro-inflammatory cytokine production[31]. With reference to biodegradability, the use of PLGA nanoparticles as a vaccine carrier has been investigated through oral administration in fish such as rainbow trout[32,33].

The innate immune system can recognize and differentiate the unmethylated oligonucleotides containing CGdinucleotides-CpG motifs (CpG ODNs) from the vertebrate's methylated DNA with the help of pattern recognition receptors (PRRs) which trigger immune response against the perceived threat of bacterial infection[34]. Recently CpG ODN motifs of bacterial DNA are recognized as a new class of adjuvants[35]; since they can induce a strong humoral and cellular immune response with a bias towards a T-helper type 1 (Th1) response[36]. CpG ODN treatment stimulated leucocyte immune activities in teleost fishes[37,38]. However, there is no report of PLGA encapsulated within CpG ODN in fish through intra-peritoneal administration in fishes. Hence the present study was undertaken to evaluate the efficacy of PLGA nanoparticles encapsulated CpG ODN nanospheres on innate and adaptive immune responses in *C. carpio* against *A. hydrophila* infection.

MATERIALS AND METHODS

Chemicals

ODNs sequence of ODN 1668 (52 - TCCATGACGTTCCTGATGCT-32) were synthesized by Bioneer Corporation, Korea and the nucleotides were underlined as indicate phosphorothioate linkage. PLGA 50:50 co-polymer (inherent viscosity 0.17 dl/g in hexafluroisopropanol) was purchased from Birmingham Polymer Inc. (Birmingham, AL, USA). Polyvinyl alcohol (PVA) (87-89% hydrolyzed, 31000-50000 g/mol) was purchased from Merck (Darmschtadt, Germany). The organic solvents used were of HPLC grade.

Preparation of PLGA-encapsulated CpG ODN nanospheres

PLGA microparticle encapsulated CpG ODN

1668 nanospheres (NS) was formulated using a double emulsion-solvent evaporation technique[39] with small modification. Briefly, 50 ug of ODN 1668 dissolved in 500 µl of phosphate buffered saline (PBS, pH 7.2) was emulsified with 150 mg of PLGA in chloroform solution (5% w/v) followed by vortexing for 3 min to get a primary emulsion. The primary emulsion was further emulsified in an aqueous PVA solution (15 ml, 5% w/v) to form an oil-in-water emulsion. For preparation of nanospheres, the emulsion was homogenized for 3 min and stirred overnight at room temperature to allow the evaporation of organic solvent. Nanospheres were recovered by normal centrifugation at 5000 xg for 20 min (SIGMA 3K30, Germany). The process of centrifugation was repeated three times to remove excess PVA and un-encapsulated CpG ODN. The recovered nanospheres suspensions were lyophilized for two days (-80 °C and <10 mm mercury pressure (LYPHLOCK, Labconco, Kansas City, MO) to get lyophilized powder for further use. PLGA microparticle encapsulated CpG ODN nanospheres was performed by scan electron micrograph as shown in Figure 1.

NS characterization

NS morphology and particle size measurement

The NS morphology was determined by scanning electron microscopy (SEM). The size and size distribution of the NS were analyzed by a laser light-scattering method (Mastersizer, Malvern Instruments, UK). Particle size distributions were calculated in the volume-weight mode and characterized as mean diameter, and were used to determine the span of the distribution.

Zeta (ζ) potential analysis

The zeta (ζ)-potential was determined in a Zeta Plus Potential Analyzer (Brookhaven Instruments Corporation, New York, USA).

Encapsulation efficiency

Twenty milligrams of NS were dissolved in 0.1 M acetic acid for 18 h, and the sample was then centrifuged for 20 min at 20,000 xg. The protein was measured by a spectrophotometric assay, with a Quanti Pro BCA assay kit (Sigma Aldrich).

Coupling efficiency

The amount of protein in the NS was quantified with a Quanti Pro BCA assay. The amount of unbound ODN recovered by centrifuging and washing the NS was subtracted from the initial amount of protein. The fluorescence was also measured with Fluorostar Optima equipment (BMG Labtech, Biogen, Madrid, Spain) at 485 nm fluorescence excitation and 520 nm for emission and wavelength, respectively.

In vivo experiment

Aeromonas hydrophila

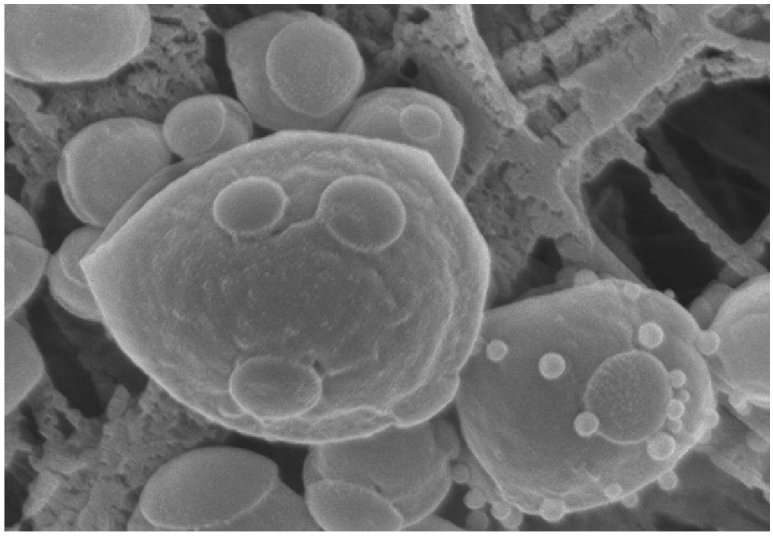

Figure 1 : PLGA nanoparticle-encapsulated CpG ODN nanospheres performed by scan electron micrograph

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A. hydrophila was associated from bacterial haemorrhagic septicemia of goldfish (*C. auratus*) in India and maintained in the laboratory[40]. It was cultured in extract-peptone medium broth at 27 °C overnight, then sub-cultured into new medium for 12 h. Then the bacteria were washed with PBS and re-suspended in PBS prior to use. The desired number of bacteria was adjusted by measuring the optical density at 600 nm, corresponding to $1.3x10^8$ cells ml^{-1}.

Administration protocol

Healthy common carp, *Cyprinus carpio*, average weight ranging from 29 to 35 g was purchased from commercial fish farm and acclimatized with 500 L aerated cement tanks for 2 weeks in the wet laboratory. One-third of the water was exchanged daily and the water temperature, pH, dissolved oxygen, and salinity were measured at 28.8 ± 1.5 °C, 7.6 ± 0.7, 5.61±0.64 mg L^{-1}, and 31.1 ± 1.1‰, respectively during the experimental period. For the immunological assay, the fish were divided into three groups of 25 each in triplicate set and injected separately each with 100 µl of PLGA, CpG ODN 1668, and PLGA-encapsulated ODN 1668 (PLGA+ODN 1668) nanospheres. After 3 week post-injection all groups were injected intra-peritoneally (i.p.) with 100 µl PBS containing *A. hydrophila* at $1.3x10^8$ cells ml^{-1}. Another two groups of 25 fish each were maintained separately for controls and injected with 100 µl of PBS (C) or bacteria (I). Six fish were randomly collected in each treated groups including the control groups and 0.5 ml of blood was collected from vein at weeks 1, 2, and 4 post-infection after anaesthetised with MS-222 ($NaHCO_3$ and tricaine methanesulphonate; Sigma Chemicals) 1:4000 in dechlorinated water for immunological assay. Only 20 fish in each group were used separately for the challenge study and record the cumulative mortality and relative percent survival (RPS) over a period of 30 days[41].

Preparation of anti-carp-globulin mice serum

The rabbit anti-carp globulin was prepared by the following method of Swain et al.[42] using sera obtained from healthy adult carp. The serum was collected from healthy carp pooled and added with an equal volume of saturated ammonium sulphate solution. They were mixed with the pooled sera drop by drop and then placed on a magnetic stirrer overnight at 4 °C. The sample mixture was centrifuged at 10,000 xg for 10 min at 4 °C and the precipitate was dissolved with 5 ml carbonate-bicarbonate buffer (pH 9.6). Then the sera were centrifuged at 10,000 xg for 10 min at 4 °C. Then pellet was collected and the volume was made to 2 ml with carbonate-bicarbonate buffer (pH 9.6). The globulin solution was dialyzed using dialysis membrane (Snakeskin, Pierce Chemical Company, USA) with 7000 molecular weight cut off against PBS (pH 7.2) for 72 h at 4 °C. After the globulin was collected and raised in a mice by the following method of Lund et al.[43].

Immunological assays

The myeloperoxidase activity was determined with 15 µl of serum and diluted in 135 µl of Hank's balanced salt solution (HBSS; Ca^{2+}, Mg^{2+} free). After this 50 µl of 20 mM, TMB (3, 30,5,50-tetra methyl benzidine) and 5 mM H_2O_2 were added. The reaction was stopped after 2 min by adding 50 µl of 4 M sulphuric acid and the optical density (O.D.) was read at 450 nm[44] using UV-VIS spectrophotometer (Thermo Spectronic, UK). The respiratory burst activity was measured by the reduction of nitro blue tetrazolium (NBT) by intracellular superoxide radicals[45]. For bacterial agglutination test, two-fold serial dilutions of 25 µl fish serum was made and an equal volume of PBS was added in each well; then addition of 25 µl of formalin killed *A. hydrophila* ($1.3x10^8$ cells ml^{-1} suspension) in 'U'-shaped microtitre plates according to Behera et al.[33]. The haemagglutination activity of serum samples was carried out using a standard methods of Blazer and Wolke[46] and Behera et al.[33] using 'U'-shaped microtitre plates by two-fold serial dilution. The haemolytic titre of serum was determined as described previously[46] and Behera et al.[33] in haemoagglutination titre by using fresh sera from all the groups.

Statistical analysis

The statistical analysis system (SAS) software (version 6.12) was used to analyse each data. One-

way analysis of variance followed by Duncan's multiple range test were done to compare the variations in various immune parameters at $p < 0.05$ significance level difference between the injected groups. The mean standard error (± S.E) of assayed parameters was calculated for each group of fish.

RESULTS

Particle size, ζ-potential, morphology, coupling efficiency of PLGA/ODN NS

The best formulation for *in vivo* studies was selected on the basis of the coupling efficiency results. To assess the particle size and the ζ-potential after ODN coupling, both parameters were measured before and after coating. The particle size and ζ-potential of the PLGA/ODN NS are presented in TABLE 1. The present study there were no significant differences between the mean size of NS of the formulations, and incorporation of the ODN did not lead to an increase in the mean diameter of the NS. Furthermore, ODN encapsulation did not have a significant impact on particle size. As expected, the ζ-potential of particles was clearly positive for non-coated and coated PLGA/ODN nanospheres. The ζ-potential varied when the NS were coated with ODN, confirming the surface modification of PLGA/ODN NS (TABLE 1) due to the polycationic charge of PLGA and ODN. The surface and shape of NS were also examined by SEM as shown in Figure 1. Topology and size of the microparticle as observed by SEM analysis confirmed the smooth and spherical nature of ODN-loaded PLGA NA.

Mortality

The least cumulative mortality of 10% (RPS: 88.9%) was observed in fish injected with ODN

TABLE 1 : Variation of size, zeta potential, and coupling efficiency.

	Size (µm)	Zeta (ζ) potential of PLGA-ODN NS	Coupling efficiency (µg/mg of NS)
Without PLGA & ODN	1.12	+32.12±0.18	-
PLGA	1.32	+33.27±0.23	38.3±0.5
PLGA containing ODN	1.44	+34.34±0.36	40.8±0.7

TABLE 2 : Cumulative mortality[a] and relative percent survival of kelp grouper after immunization with PLGA and ODN 1668

Groups	Tank	Cumulative mortality (%)	Mean cumulative mortality (%)	RPS[b] (%)	Statistical ignificance (χ^2 test)[c]
Control	A	20/20=100	100		
	B	20/20=100			
	C	20/20=100			
Infected	A	16/20=80	90		
	B	18/20=90			
	C	20/20=100			
PLGA	A	3/20=15	15	83.3	P<0.05
	B	2/20=10			
	C	4/40=20			
ODN	A	1/20=5	10	88.9	P<0.05
	B	3/20=15			
	C	2/20=20			
PLGA+ODN	A	1/20=5	10	88.9	P<0.05
	B	4/20=20			
	C	1/20=5			

[a]Cumulative mortality was calculated control and immunized groups; [b]RPS={1-(% mortality of immunized group/% mortality of non-immunized group)}x100; [c]Statistical significance is based on a comparison to results for the non-immunized group.

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1668 and PLGA-encapsulated ODN 1668 nanospheres during a period of 30 days and in PLGA group the mortality was 15% (RPS: 83.3%). In infected and non-injected group the cumulative mortality was 90% while no mortality was observed in control group (TABLE 2).

Myeloperoxidase (MPO) activity

The myeloperoxidase (MPO) activity did not significantly vary in any experimental group on first week when compared to the control against pathogen. Fish injected with PLGA-encapsulated ODN 1668 nanospheres had significantly increased MPO activity on week 2 which was not observed in other groups. On week 4, fish injected with ODN 1668 and PLGA-encapsulated ODN 1668 nanospheres had significantly increased MPO activity against pathogen; however, the activity did not significantly increase in PLGA group on week 4 (Figure 2).

Respiratory burst (RB) activity

The respiratory burst (RB) activity was not significantly enhanced PLGA administered group from weeks 1 to 4; however with ODN 1668 and PLGA-encapsulated ODN 1668 nanospheres it significantly enhanced from weeks 1 to 4 (Figure 3).

Serum haemoagglutinating titre

The serum haemoagglutinating titre did not significantly increase in any experimental group on first week when compared to the control. The titre significantly increased more with PLGA-encapsulated ODN 1668 group on week 2 than with PLGA or ODN 1668 alone. On the other hand, it significantly in-

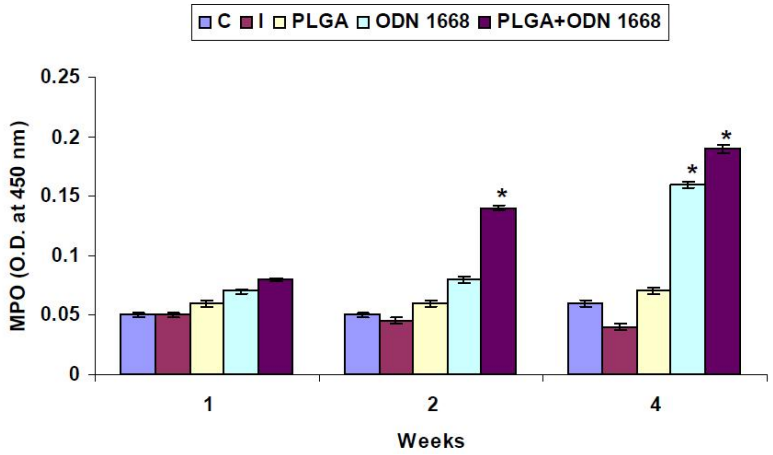

Figure 2 : Myeloperoxidase (MPO) activity of *C. carpio* (n = 6) control (C), infected (I), and injected with PLGA, ODN 1668, and PLGA-encapsulated ODN 1668 against *A. hydrophila*. Data (mean ± SE; *P < 0.05) difference from the control is indicated with asterisks

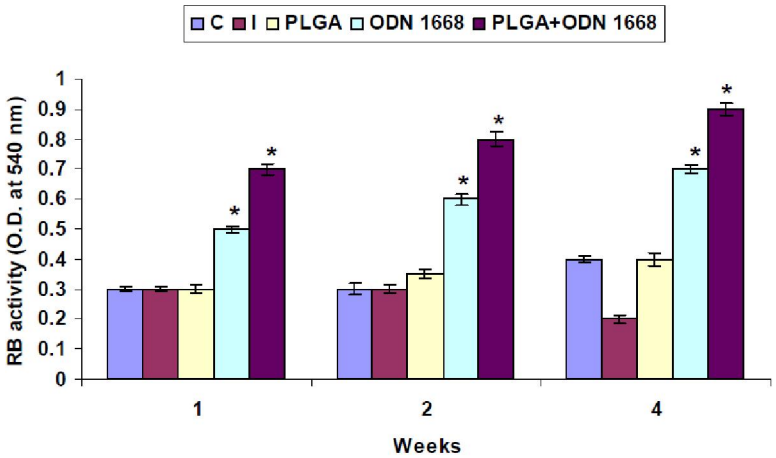

Figure 3 : Respiratory burst (RB) activity of *C. carpio* (n = 6) control (C), infected (I), and injected with PLGA, ODN 1668, and PLGA-encapsulated ODN 1668 against *A. hydrophila*. Data (mean ± SE; *P < 0.05) difference from the control is indicated with asterisks

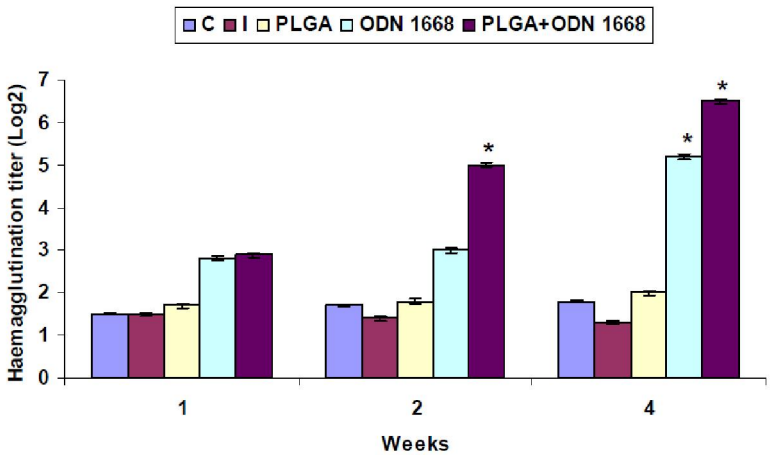

Figure 4 : Serum haemoagglutinating activity (titre) of *C. carpio* (n = 6) control (C), infected (I), and injected with PLGA, ODN 1668, and PLGA-encapsulated ODN 1668 against *A. hydrophila*. Data (mean ± SE; *P < 0.05) difference from the control is indicated with asterisks

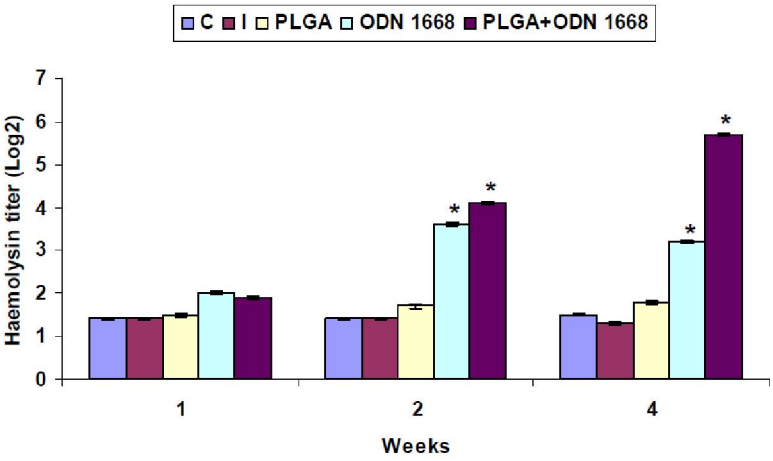

Figure 5 : Serum haemolysin activity (titre) of *C. carpio* (n = 6) control (C), infected (I), and injected with PLGA, ODN 1668, and PLGA-encapsulated ODN 1668 against *A. hydrophila*. Data (mean ± SE; *P < 0.05) difference from the control is indicated with asterisks

creased in ODN 1668 alone or PLGA-encapsulated ODN 1668 nanospheres on week 4 against pathogen (Figure 4).

Serum haemolysin titre

In all the groups, the serum haemolysin titre did not enhance significantly on first week when compared to the control against pathogen. On weeks 2 and 4, it was significantly enhanced in fish injected with ODN 1668 and PLGA-encapsulated ODN 1668 nanospheres but not in PLGA alone (Figure 5).

Bacterial agglutination titre

The bacterial agglutination titre did not significantly increase in any group on first week. On the other hand, the bacterial agglutination titre significantly increased in fish injected with ODN 1668 alone or PLGA-encapsulated ODN 1668 nanospheres against pathogen on weeks 2 and 4. However, there was no significant difference in bacterial agglutination titre in fish injected with PLGA alone (Figure 6).

DISCUSSION

CpG ODN could impart particulate nature to soluble antigens and increase their interaction with APCs and macrophages[39]. It can deliver peptide antigens to APCs[47] and generate Th1 type immune response, even against poor immunogens[48]. They have been used to co-encapsulate the antigen(s) which serve as an adjuvant to deliver the antigen and stimulatory adjuvant to the same APC; it induces

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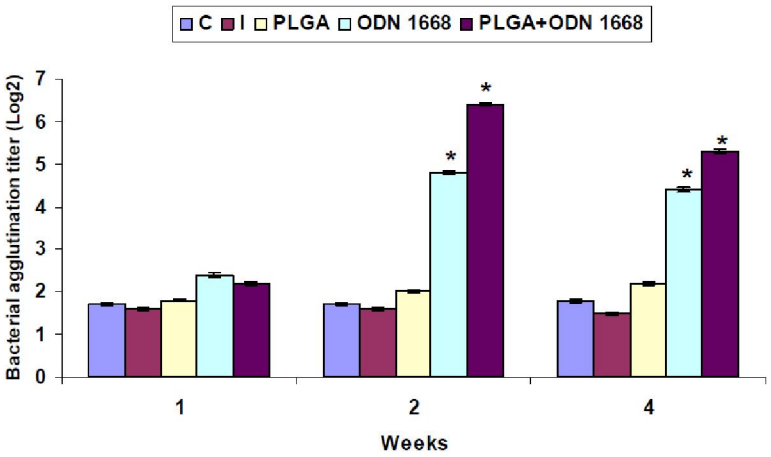

Figure 6 : Bacterial agglutination activity (titre) of *C. carpio* (n = 6) control (C), infected (I), and injected with PLGA, ODN 1668, and PLGA-encapsulated ODN 1668 against *A. hydrophila*. Data (mean ± SE; *P < 0.05) difference from the control is indicated with asterisks

stronger immune response when compared to the free antigens and adjuvants[39]. CpG ODN motifs act as an immune adjuvant inducing strong humoral and cellular immune responses with a bias towards a Th1 response[49,50].

NS consisting of biodegradable polymer particles represent a promising antigen or protein delivery system and slow release for immunotherapy. It was prove a number of advantages over conventional delivery systems. For example, the microencapsulation has been acts in an adjuvant capacity via increased uptake by APCs[51] and the diameter of the NS produced in this work (1 to 10 μm) was adequate to allow their uptake by professional APC[52]. In the present study, two types of NS formulations were tested: (i) PLGA and PLGA-encapsulated ODN biodegradable NS. Our main objective was to determine the immunogenicity of NS with ODN preparations, using fish as experimental model. In the present study there were no significant differences between the mean size of NS of the formulations, and incorporation of the ODN did not lead to an increase in the mean diameter of the NS. In addition PLGA-encapsulated ODN biodegradable NS did not have a significant impact on particle size as expected of the particles was clearly positive for non-coated and coated PLGA/ODN nanospheres.

In the present study show that PLGA-encapsulated ODN 1668 resulted in a significant increase in the MPO activity on week 2 whereas with ODN 1668 alone and PLGA-encapsulated ODN 1668 groups the activity increased only on week 4. It is well known that PLGA has been reported to act as a very useful antigen delivery system in mammal since it provides long lasting immunity[53]. The humoral immune response of mice to PLGA-MS, on receiving a single injection of MS containing OVA and CpG oligonucleotides (MS-OVA-CpG) indicates a robust IgG1 and IgG2a response suggesting a balanced Th1/Th2 response. The enhanced response after heterologous as opposed to homologous boosting with MS-OVA/CpG, the potential of PLGA-MS- and IFA-based vaccination induced *in vivo* proliferation of OVA-specific $CD8^+$ and $CD4^+$ T cells derived from OT-1 and OT-2 mice at several time points after vaccination has been reported[54]. In the present study, the MPO activity did not significantly increase in any experimental group on first week. The results are in agreement when PLGA-MS containing either tPrP or CpG-ODN alone or in combination which failed to induce detectable immune response in treating cancer[54].

With respect to the immune response of the formulations, the NS containing NS covalently coupled to the surface induced respiratory burst activity was more significantly enhanced in ODN 1668 alone and PLGA-encapsulated ODN 1668 nanospheres from weeks 1 to 4 than with PLGA in this study. The haemoagglutinating titre was significantly enhanced with PLGA-encapsulated ODN 1668 on week 2 while with ODN 1668 and PLGA-encapsulated ODN 1668 groups the increase manifested on week

4. However, the formulations of NS with encapsulated ODN induced almost no serum haemoagglutinating titre in any group on first week. Comparable results have also been reported by the use of PLGA alone in mammal[47] as well as in fish like rainbow trout[55]. Similarly Japanese flounder after immunization with PLGA-encapsulated DNA vaccine had increased immune response such as Superoxide dismutase assay (SOD) and respiratory burst activities against lymphocystis disease virus (LCDV)[56]. In grass carp after administration with ODN-1826 and -2006, ODN-1670, and ODN-D could activate macrophages, increasing the levels of superoxide anion[57]. The efficacy of PLGA particles as antigen carrier has been evaluated indicating high potencies in activating the adaptive immune response in mammals[58]. Stimulation of the immune system has been investigated after intramuscular and intradermal administration of free or lipid complexed CpG ODN alone or in combination with peptide, protein or DNA vaccination[59,60]. A recent study indicates that CpG-ODN 1668 might activate an alternative pathway of complement in olive flounder, which is an important innate immune factor in conferring resistance against *P. dicentrarchi* infection[61]. In rainbow trout the serum lysozyme activity was enhanced when injected with 10 mg and 3.16 mg of CpG ODN 2133[62]. In common carp treated with CpG ODN B and C activated macrophages, increasing the level of production of superoxide anion and phagocytic activity[63].

The haemoagglutinating titre was significantly enhanced in PLGA-encapsulated ODN 1668 on week 2 and ODN 1668 and PLGA-encapsulated ODN 1668 nanospheres on week 4 but not in any other group on first week. The serum haemolysin titre was significantly enhanced in ODN 1668 and PLGA-encapsulated ODN 1668 groups on week 2 and 4 but not in PLGA. Similar results were also found when PLGA was used as carrier for peptide vaccine in mammals[64]. Moreover, the superiority of PLGA microspheres over alum adjuvant in eliciting high antibody and immune response responses was seen in mice through subcutaneous administration[65]. The present results are in agreement with that of rainbow trout injected with 10, 1.0, and 0.1 mg of CpG-ODN 2133 which induced a higher antibody titre[66]. Atlantic salmon after treatment with PLGA incorporated human gamma globulin or antigen[67] and Japanese flounder after treatment with PLGA-encapsulated plasmid vaccine against LCDV[32,69] also produced similar results. According to O'Hagan et al.[68], the antibody levels remained high even one year after subcutaneous injection in mice which indicate that the injectable PLGA microparticles control the release of antigen over a period of several weeks. CpG motifs co-encapsulated into PLGA 502 induced higher antibody titres and increased production of IgG2a antibodies that OVA microencapsulated alone, being more effective that both co-administered freely in solution. On the contrary, co-delivery of CpG and OVA in PLGA 756 microparticles decreased IgG2a improved antibody response in mice immunized with OVA CpG PLGA 502 in comparison with CpG simply co-administered with the antigen[70].

The bacterial agglutination titre significantly increased in fish injected with ODN 1668 and PLGA-encapsulated ODN 1668 nanospheres on weeks 2 and 4. In the present study administration with PLGA-encapsulated ODN 1668 nanospheres showed encouraging results without any adverse effects on fish health. Correspondingly the cumulative mortality was 10% in ODN 1668 and PLGA-encapsulated ODN 1668 nanospheres while it was 15% in PLGA indicating their potential role in fish disease management in aquaculture. Similarly, olive flounder injected with a high dose of CpG ODN 1668 or GpC-ODN 1720 suffered a high mortality of 70%[61]. In Nile tilapia immunized with PBS, PBS+ODN 21 and PBS+ODN 2007 the cumulative mortality ranged from 70 to 60%; on the other hand administration of QCDCR, and QCDCR+ODN 18S, inflicted a cumulative mortality was 80% each; but with QCDCR+ODN 2007 the observed mortality was reduced to 20%[71] indicating the species specific result as a function of vaccine and the dosage. In rainbow trout the CpG-ODNs significantly enhance the survival against bacteria[30]. Olive flounder administered with CpG-ODN 1668 showed a significantly higher survival against *M. avidus* infection[61]. Later, Jørgensen et al.[72] reported *in vivo*

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enhancement of resistance against IPNV challenge in Atlantic salmon by i.p. injection of CpG-ODN 1681 (B-class ODN). Biochemical properties, as well as other parameters, such as release and stability, must be determined for each preparation so that each new batch of PLGA-encapsulated NS is characterized. The present study indicates that single injection with PLGA-encapsulated ODN 1668 nanospheres elicits better immune response than those of PLGA or ODN 1668 alone in *C. carpio* against *A. hydrophila*. This was agreement with PLGA microparticles as a vaccine carrier can reduce the number of administrations and induce both innate and adaptive immunity in rohu[33]. Although the mechanisms responsible for the protective effects of this NS in fish are not well understood, the fact that the components used in its formulation are already approved for clinical use favours use of this ODN in the development of a more effective and safer immunostimulant against disease. Further detailed molecular and immunological studies are necessary to confirm the efficacy in other fishes.

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