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Nanoconjugation of bicistronic DNA vaccine against *Edwardsiella tarda* using chitosan nanoparticles: Evaluation of its protective efficacy and immune modulatory effects in *Labeo rohita* vaccinated by different delivery routes

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ABSTRACT

DNA-based immunization has proven to be an effective prophylactic measure to control aquatic animal diseases. In order to improve the efficiency of vaccine against fish pathogen, novel delivery mechanism needs to be adopted. In the present study we nanoconjugated the previously constructed DNA vaccine (pGPD + IFN) with chitosan nanoparticles (CNPs) by complex coacervation process. After construction of the vaccine, an *in vivo* vaccination trial was conducted in which 2 groups of rohu (*L. rohita*) fingerlings were vaccinated with CNPs-pGPD + IFN, one group by oral route (incorporated in feed for 14 days) and the other by immersion route (primary and booster immunised), whereas, a third group was intramuscularly (I/M) injected (initial and booster immunised) with naked pGPD + IFN and subsequently challenged with *E. tarda* (8.7×10^4 CFU/fish) at 35-day post initial vaccination. The protective immune responses were determined in terms of relative percentage survival (RPS), specific antibody production, non-specific immune response, expression kinetics of immune-related genes and pathological manifestation. Evaluation of RPS analysis revealed that CNPs-pGPD + IFN groups recorded highest RPS (81.82% and 72.73% in oral and immersion vaccinated fish group respectively) while the naked pGPD + IFN injected group showed 63.62% RPS when compared with 55% cumulative mortality of control group. In addition, NBT, myeloperoxidase activity, serum lysozyme activity and specific antibody titre in case of CNPs-pGPD + IFN groups showed higher activities during all the time points. Furthermore, CNPs-pGPD + IFN groups showed significant ($p < 0.05$) upregulation of different immune gene transcripts (IgHC, iNOS, TLR22, NOD1 and IL-1β) in three immunologically important tissues post immunization (both primary and booster dose) as well as after challenge. Thus, from this study, we can conclude that oral or immersion vaccination with CNPs-pGPD + IFN can orchestrate an effective immunisation strategy in organizing a coordinative immune response against *E. tarda* in *L. rohita* exhibiting minimum stress to the host with maximum efficacy.

1. Introduction

Indian major carps (IMCs) constitute the mainstay of Indian aquaculture with production over two million tonnes per year. However, this increase in production has led to occurrence of infectious diseases causing substantial losses to the carp farming. Thus the need for protection of the highly intensive culture system from infectious disease has becomes priority among fishers community. Among the various bacterial pathogens causing economic losses to the Indian aquaculture industry, *Edwardsiella tarda (E. tarda)*, a gram negative, facultative anaerobic bacterium belonging to the family Enterobacteriaceae, causes Edwardsiellosis/putrefactive systemic infection in both marine and freshwater fishes [1]. Pathological manifestation associated with the disease are distended

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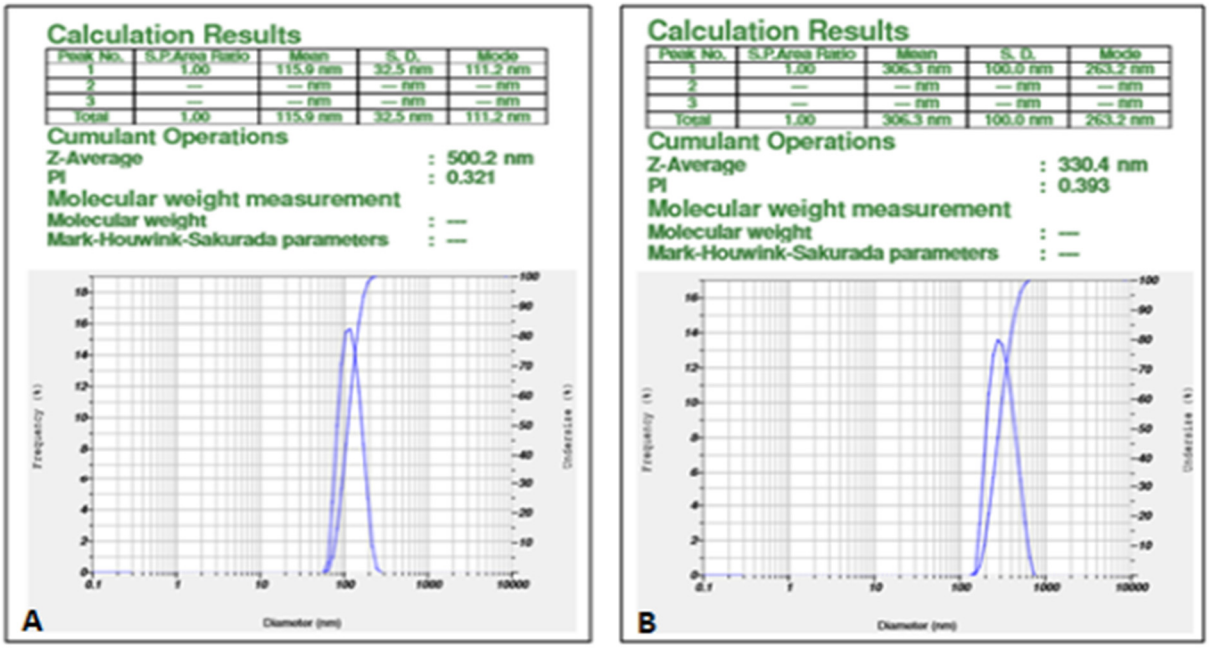

Fig. 1A. Particle size distribution of Chitosan Nanoparticles (CNPs) by dynamic light scattering (DLS) using HORIBA Scientific Nano particle analyzer SZ-100. (A) Blank CNPs; (B) CNPs-pGPD + IFN.

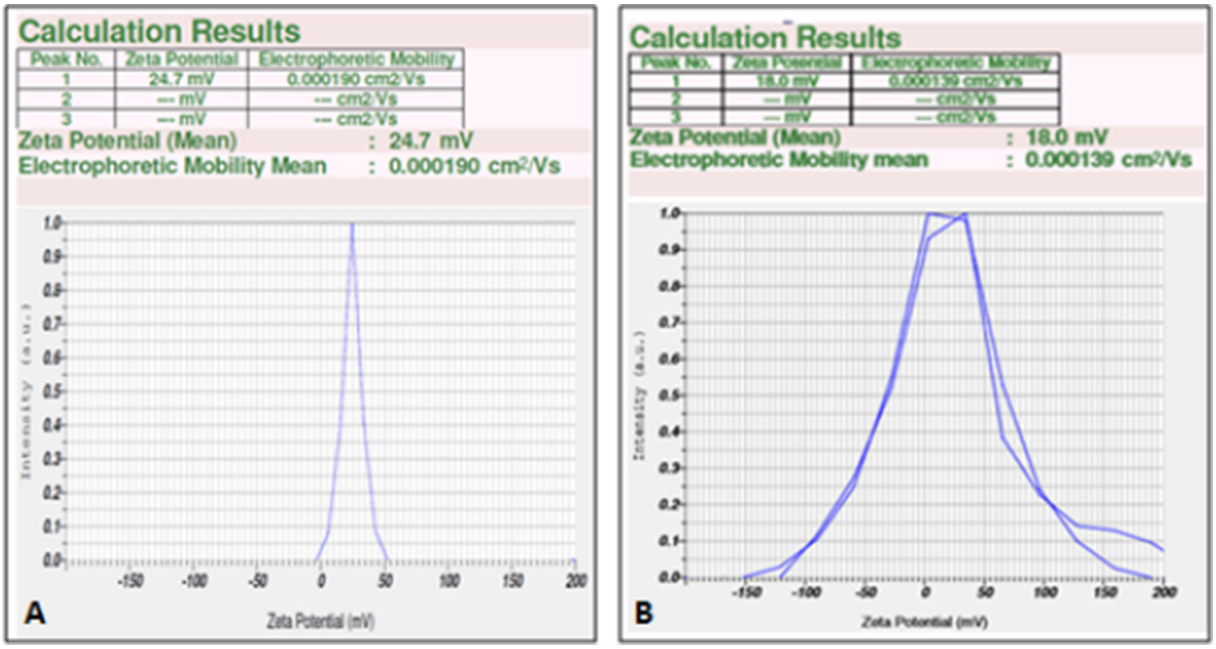

Fig. 1B. Zeta Potential of Chitosan Nanoparticles (CNPs) using HORIBA Scientific Nano particle analyzer SZ-100. (A) Blank CNPs; (B) CNPs-pGPD + IFN.

abdomen, prolapsed rectum, cutaneous lesions inside the musculature, fibrinous peritonitis along with necrosis of the hepatic and nephritic tissues [2].

In an attempt to control the spread of this disease, we previously developed a bicistronic DNA vaccine (designated as pGPD + IFN) containing a regular antigenic gene (glyceraldehyde-3-phosphate dehydrogenase gene of *Edwardsiella tarda*) along with an additional immune adjuvant gene (Interferon gamma gene of *Labeo rohita*) [3]. The vaccines construct was found to be successfully expressing the antigenic proteins both *in vitro* and *in vivo* and eliciting higher protective immunity in *L. rohita* against virulent *E. tarda* challenge but the mode of administration was intramuscular injection. Thus in lieu of the protective efficacy exhibited by the constructed vaccine, we presently focused on replacing the injection route of vaccine delivery by oral and immersion routes as the parental immunization method involves several limitation including stress on the fish, labour intensiveness, time requirements, unsuitability for administration in large number of small fish (<20 g) which are most susceptible to bacterial infection along with safety issues for fish as well as administrators [4].

Oral and bath immunization are attractive option to cater the disease outbreak, which offers convenience for mass vaccination and the advantage of zero handling stress to fish [5]. However, in contrast to the injection method, vaccine delivered through oral route,

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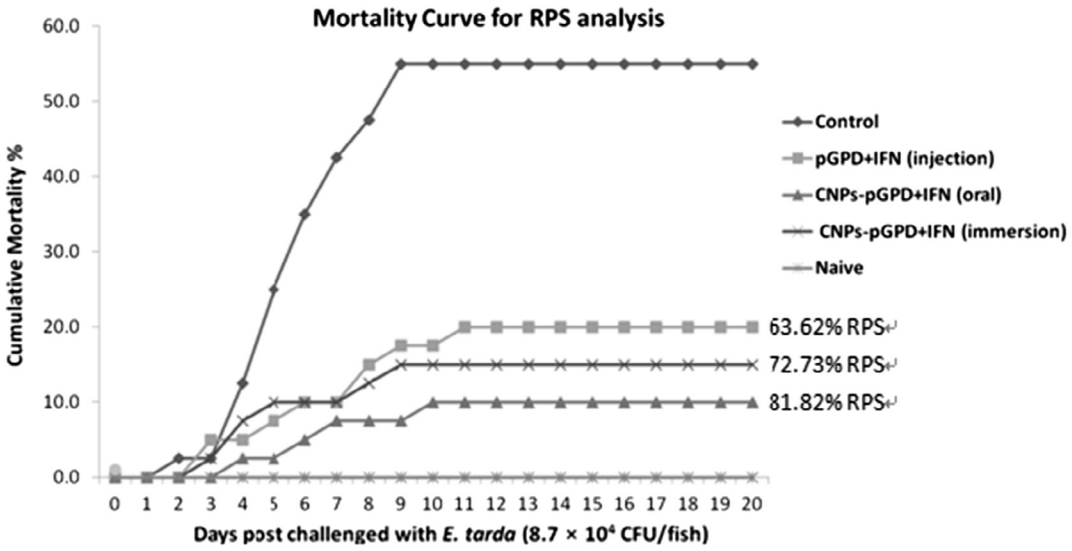

Fig. 2. Cumulative mortality and relative percentage survival (RPS) analysis of different experimental groups (pGPD + IFN (injection), CNPs-pGPD + IFN (oral), CNPs-pGPD + IFN (immersion) and Control) of *L. rohita* (n = 40, 2 tanks with 20 fish/tank for each groups) challenged with *E. tarda* (8.7×10^4 CFU/fish) at different time intervals. Naive group was kept unchallenged.

needs to be protected against degradation in the hostile gut environment before they effectively delivered to immune cells where immune induction occurs [6], whereas, the efficacy of bath immunization is sometimes affected due to several barriers of skin and gill epithelium [7]. Also, both oral and immersion method of immunization require more quantity of target vaccine, especially for large fish. Hence, for replacing injection route by oral or immersion method, it is necessary to overcome these obstacles and develop a simple, cost effective and efficient vaccine delivery strategy.

In this regard, nano-delivery of vaccines, which can provide high efficacy with least side effect, has been explored in recent years. Nanoencapsulation or nanoconjugation increases the stability of the encapsulated or conjugated biomolecules by protecting it from the hostile *in vivo* environment and facilitates sustained release of the drug [8]. Recently, a number of novel techniques have been developed to introduce a foreign DNA into cells. Among various non-viral gene transfer agents, chitosan have received much attention because of its excellent biocompatibility, low immunogenicity, and reduced cytotoxicity compared to other polymers [9,10]. It is considered as a good candidate for gene delivery system as positively charged chitosan can be easily complexed with negatively charged DNA [11]. Previous studies have also reported the feasibility of gene transfer into fish by encapsulating the DNA into chitosan and incorporating into fish feeds [12].

Thus, in the present study, we conjugated the bicistronic DNA vaccine (pGPD + IFN) with chitosan nanoparticles (CNPs) by complex coacervation process [13] to prepare a nanoconjugated DNA vaccine for non-stressful delivery strategy. Further, the nanoconjugated DNA vaccine was administered to *L. rohita* by oral and immersion routes to evaluate their effects in triggering immune response in the host as well as their protective immunity for combating *E. tarda* infection by challenge study. In addition, a comparative analysis of the immune response induced by the nanoconjugated vaccine (delivered by oral and immersion routes) against the naked vaccine (injected intramuscularly) was also evaluated.

2. Material and methods

2.1. Experimental animals

Rohu (*L. rohita*) juveniles (25.0 ± 2.6 g) obtained from a local farm were stocked into 500 L fibre reinforced plastic (FRP) tanks with aerated freshwater in an indoor rearing facility for 3-weeks prior to the immunization trial and were fed twice a day with a standard pelleted diet at 3% of their body weight. Water quality of the tanks was maintained regularly. The water temperature varied from 27 °C to 28 °C and the pH of the water was in the range of 7.4–7.6 during the experiment. To confirm the fishes were *E. tarda*-free, bacterial isolation in SS-agar (Salmonella-Shigella) plate from kidney of ten randomly selected individuals was carried out.

2.2. Bacterial strains

Edwardsiella tarda used in the study was obtained from ATCC, USA. *Edwardsiella tarda* ATCC ® 15947™ was revived using brain heart infusion (BHI) broth from cult loop. The broth was incubated for 18–24 h at 37 °C. The bacterial culture was then streaked on SS-agar plate and incubated at 28 °C. Single black colony was grown in BHI broth at 28 °C for 22 h for challenge study.

2.3. Extraction of plasmid DNA construct (pGPD+IFN)

The plasmid (pGPD + IFN) constructed previously [3] was isolated using the QIAGEN Plasmid Maxi Kit (QIAGEN, Germany) as per the manufacturer's protocol. The concentration of the recombinant plasmids was measured using Nanodrop spectrophotometer (Thermofisher Scientific, USA) and diluted with nuclease free water (NFW) to 100 ng/μL concentration.

2.4. Preparation of chitosan nanoparticles (NPs)

The chitosan NPs was prepared by following the method as described previously [13] (Detailed procedure in Supplementary file 1).

2.5. Determination of zeta potential and size of nanoparticles

The prepared chitosan NPs were characterized in terms of size, size distribution, and zeta potential by dynamic light scattering (DLS) using HORIBA Scientific Nano particle analyzer SZ-100. Briefly, 1.3 mL of sample at a concentration of 0.3 mg/mL was placed in a polystyrene cuvette and measured at 25 °C. The viscosity and refraction index were set equal to those specific to water.

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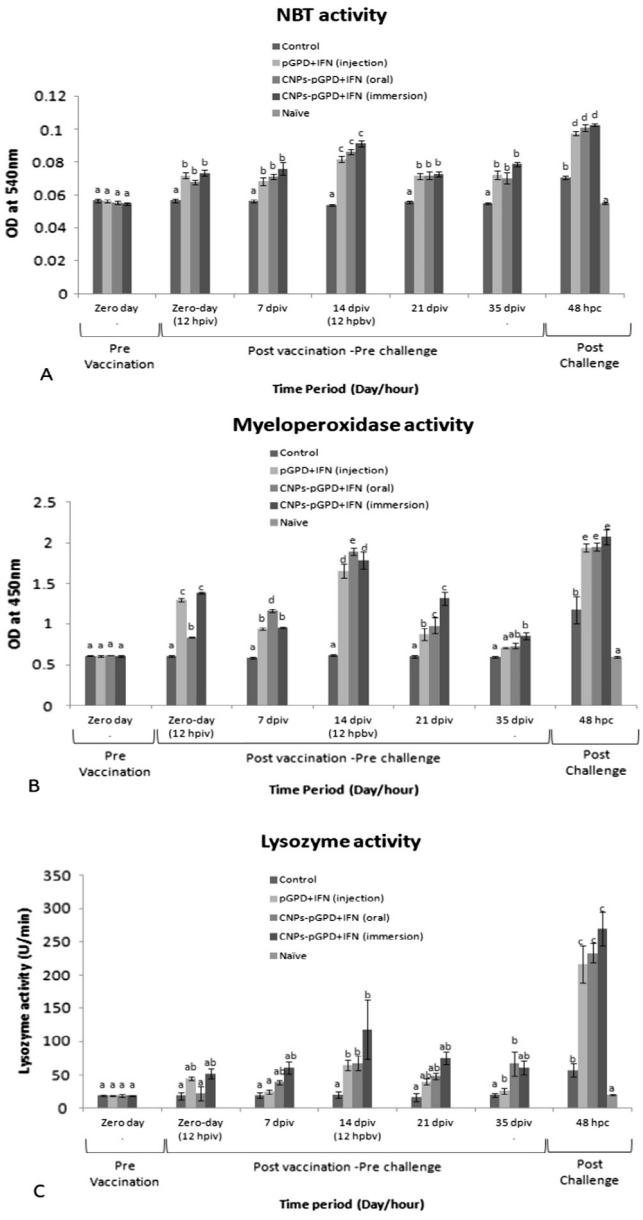

Fig. 3. Non-specific immune responses of different experimental group viz. pGPD + IFN (injection), CNPs-pGPD + IFN (oral), CNPs-pGPD + IFN (immersion), Control group and Naive group at different time intervals pre immunization, post immunization and 48 h post challenge with *E. tarda* (8.7×10^4 CFU/fish). (A) Respiratory burst activity (NBT), (B) Myeloperoxidase activity (MPO), (C) Lysozyme activity. The mean values (n = 3) of the activities in the blood cells (for NBT) or serum (for MPO and Lysozyme) were plotted with standard error as a function time after immunization/challenge. A one-way ANOVA ($p < 0.05$) and Duncan's multiple range test (homogenous subsets indicated by alphabets) was used to determine statistical significance of various non-specific immune activities at various time points. (hpiv/dpiv – h/d post initial vaccination; hpbv – h post booster vaccination).

Zeta potential was measured with a disposable capillary cell with a volume of 1 mL after purification.

2.6. Conjugation of pGPD+IFN with chitosan NPs

Equal volume of chitosan solution (0.02% in 25 mM sodium acetate buffer, pH 5.5) and plasmid DNA solution (100 ng/μL) were taken in 15 mL tubes separately and heated to 55 °C in water-bath for 5 min. The heated chitosan was added to the heated plasmid drop-wise. The final mixture was subjected to vortexing at 1000*g* for 30 s and kept at room temperature for 30 min. The size and zeta potential of the chitosan NPs conjugated pGPD + IFN (CNPs-pGPD + IFN) was determined as described previously. The chitosan NPs conjugated plasmid was stored at −20 °C for further studies.

2.7. Gel retardation test and stability test of Chitosan- pGPD+IFN nanoparticles

For confirmation of positive conjugation of chitosan NPs and plasmid, gel retardation test was conducted. Briefly, naked plasmid, conjugated product and blank chitosan NPs were run in 1% agarose gel and the migration pattern of the particles were observed under UV-light. Stability of conjugated plasmid DNA from DNase degradation was examined using DNase I enzyme (Thermofisher Scientific, USA). Briefly, naked plasmid and conjugated product (both 3 μg) were subjected to DNase I treatment separately by incubation at 37 °C for 15 min [10]. Naked plasmid and CNPs-pGPD + IFN with and without DNase I treatment were run in 1% agarose gel to check the integrity of conjugated pDNA.

2.8. Preparation of feed for oral immunization

For oral immunization, CNPs-pGPD + IFN (dose calculated as reported previously [14]) was incorporated into feed as described previously [15] with slight modifications (Detailed procedure in Supplementary file 1).

2.9. Experimental design for immunization trial

Rohu fingerlings (25.0 ± 2.6 g) (n = 400, n = number of fish) were randomly distributed into 4 treatment groups with 80 fishes in each group and designated as pGPD + IFN (injection), CNPs-pGPD + IFN (oral), CNPs-pGPD + IFN (immersion), Control group (challenged group but without any treatment) and remaining 80 fishes were kept as naive group (without any treatment or challenge). For immunization (immunization pictures in Supplementary file 2), the pGPD + IFN (injection) group was intramuscularly (I/M) injected with 100 μL of naked pGPD + IFN (10 μg/fish) plasmid construct while in the CNPs-pGPD + IFN (immersion) group, fish were distributed into four glass-bowl (20 fish/bowl) and immersed in 2 L of water with 20 mL (1 μg/mL) of chitosan NPs conjugated pGPD + IFN (dose calculated as described previously [16]) for 2 h with vigorous aeration and transferred back to the original tank after immersion. Booster dose was administered to pGPD + IFN (injection) and CNPs-pGPD + IFN (immersion) group similarly at 14 day post initial immunization. The CNPs-pGPD + IFN (oral) group was fed with the prepared feed containing CNPs-pGPD + IFN (as described earlier) for 14 consecutive days (twice/day) at the same time when normal commercialized feed was given to the other groups. Three fish from each group (immunized groups and control group) were randomly selected for sampling at zero-day (pre-treatment and 12 h post initial vaccination), 7-day (post initial vaccination), 14-day (12 h post booster vaccination), 21-day (post initial vaccination) and before challenge (35-days post immunization). Blood, serum, kidney, liver and spleen tissue samples were collected at each time point for further analysis of immune parameters.

2.10. Challenge study

Viable count of *E. tarda* (ATCC ® 15947™) was determined as colony forming unit (CFU) following 10-fold serial dilutions and plating on nutrient agar. The LD_{50} dose was calculated following the method of Reed and Muench [17] from same group of naive fishes (10 fish per dilution). For challenge study, remaining 62 fish from each of the 4 experimental groups viz., pGPD + IFN (injection), CNPs-pGPD + IFN (oral), CNPs-pGPD + IFN (immersion) and control

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groups were intra-peritoneally injected with 100 μL of bacterial suspension (LD_{50} dose of 8.7×10^4 CFU/fish) 35 day post initial immunization while naive group remained unchallenged. For mortality observation, 40 fish per group (including naive group) were divided into 2 separate tanks (n = 20/tank where n is number of fish). Mortality pattern was observed for 20 days post infection. The cause of mortality was confirmed by re-isolating the bacteria from the kidney of dead fish. Relative percentage of survival (RPS) was calculated by the formulae [18]; Relative percentage of survival (RPS) = {1 − (%Mortality in vaccinated group/%Mortality in control group)} × 100. While from the remaining fishes in each group (including naive group) 3 fish per time-point were randomly sampled for blood, serum, kidney, liver and spleen tissue samples at 6 h, 12 h, 48 h, 96 h and 168 h post challenge (hpc).

2.11. PCR detection of GAPDH gene from immunized fish

DNA was extracted from the muscle, intestine and gill tissue (tissue specific to immunization routes) collected from pGPD + IFN (injection), CNPs-pGPD + IFN (oral) and CNPs-pGPD + IFN (immersion) groups respectively at 14-day and 35-day post initial vaccination with the DNAzol reagent (Invitrogen, USA) as per manufacturer's protocol and amplified with specific primer sets for GAPDH gene (996 bp) as described previously [3]. Amplified PCR product was run in 1% agarose gel.

2.12. Non-specific immune responses

For non-specific immune responses, respiratory burst activity was analysed from blood and myeloperoxidase activity and serum lysozyme activity were analysed from serum samples collected from all experimental groups at different time intervals as described previously [19–22]. (Detailed procedures in Supplementary file 1).

2.13. Competitive ELISA for specific antibody quantification

Specific antibody (IgM) quantification was done by competitive enzyme-linked immunosorbent assay (c-ELISA) as described by Swain and Nayak [23] with slight modification. Purified rGAPDH protein developed by Banu et al. [24] and monoclonal antibody (MAbs) raised against GAPDH protein [24] were used as antigen and competitive antibody respectively (Detailed procedure in Supplementary file 1).

2.14. Immune gene expression

2.14.1. RNA isolation and cDNA synthesis

Total RNA extraction and cDNA synthesis from the collected tissues (kidney, liver and spleen) from all experimental groups i.e. pGPD + IFN (injection), CNPs-pGPD + IFN (oral), CNPs-pGPD + IFN (immersion), control and naive groups (for naive group tissues collected post-challenge) at different time-points were performed as described in our previous study [25].

2.14.2. Absolute quantification of different genes using real-time PCR

Absolute quantification for 5 different gene transcripts (IgHC, iNOS, TLR22, NOD1 and IL-1β) present in the tissue samples from all experimental groups at different time-points was carried out in ABI 7500 Real Time PCR system (Applied Biosystems, USA) using gene specific primers as described in our previous work [25].

2.15. Statistical analysis

The data generated for different immune parameters as well as gene expressions were statistically analysed by statistical package SPSS version 22 (SPSS Inc., USA) in which data were subjected to one-way ANOVA and Duncan's multiple range test was used to determine the significant differences between the means. Comparisons were made at the 5% probability level. P value below 0.05 was considered statistically significant. The results were expressed as the mean ± S.E.

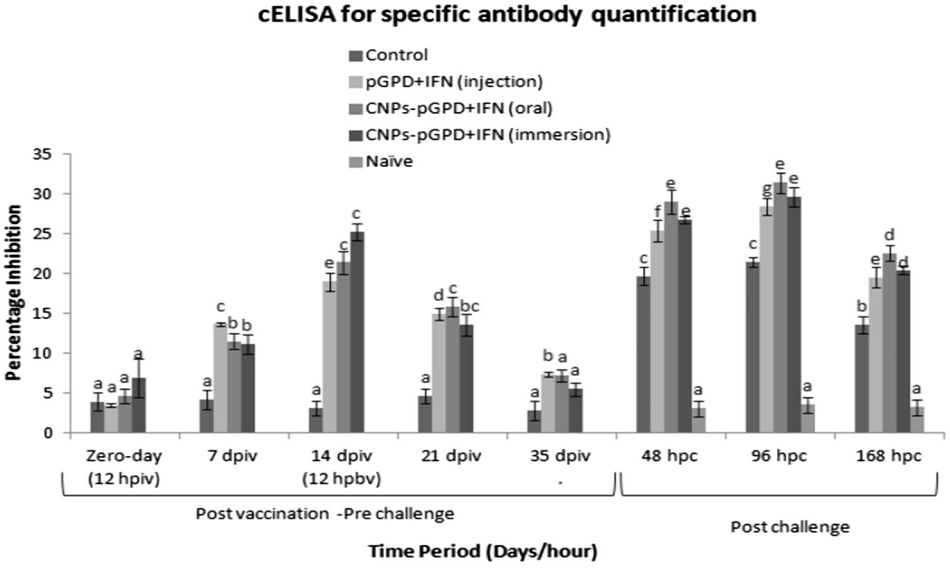

Fig. 4. Percentage inhibitions (PI) of serum-antibody against *E. tarda* were determined by competitive ELISA using anti-GAPDH MAbs. Percentage inhibition of anti-GAPDH antibody binding to recombinant GAPDH protein, present in the fish serum of different experimental group viz. pGPD + IFN (injection), CNPs-pGPD + IFN (oral), CNPs-pGPD + IFN (immersion), Control and Naive group were plotted at different time intervals post immunization and post challenge with *E. tarda* (8.7×10^4 CFU/fish). The mean values (n = 3) of the PI activities in the serum were plotted with standard error as a function time after immunization/challenge. A one-way ANOVA ($p < 0.05$) and Duncan's multiple range test (homogenous subsets indicated by alphabets) was used to determine statistical significance of the PI activities at various time points. (hpiv/dpiv – h/d post initial vaccination; hpbv – h post booster vaccination).

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Table 1
Absolute quantification of immune gene transcripts (copy number) in kidney, liver and spleen of *Labeo rohita* (n = 3) of different experimental group viz. pGPD + IFN (injection), CNPs-pGPD + IFN (oral), CNPs-pGPD + IFN (immersion), Control group post immunization. Mean ± standard error analysed per time point are displayed.

Gene		Kidney				Liver				Spleen			
	Day post vaccination	Control	pGPD + IFN (injection)	CNPs-pGPD + IFN (oral)	CNPs-pGPD + IFN (immersion)	Control	pGPD + IFN (injection)	CNPs-pGPD + IFN (oral)	CNPs-pGPD + IFN (immersion)	Control	pGPD + IFN (injection)	CNPs-pGPD + IFN (oral)	CNPs-pGPD + IFN (immersion)
IgHC	Zero-day (12 hpiv)	$40.2^{a} \pm 1.6$	$53.4^{a} \pm 7.87$	$42.94^{a} \pm 6.89$	$48.37^{a} \pm 7.51$	$14.21^{ab} \pm 1.35$	$14.99^{a} \pm 1.32$	$15.27^{a} \pm 2.72$	$14.69^{a} \pm 1.99$	$32.42^{a} \pm 2.23$	$35.72^{a} \pm 0.95$	$34.42^{a} \pm 1.73$	$34.35^{a} \pm 0.74$
	7 dpiv	$52.2^{b} \pm 2.8$	$322.8^{b} \pm 71.28$	$275.85^{b} \pm 56.11$	$333.38^{b} \pm 85.19$	$15.36^{b} \pm 1.52$	$37.82^{b} \pm 1.43$	$22.00^{a} \pm 2.11$	$26.61^{b} \pm 1.92$	$33.05^{a} \pm 0.90$	$308.78^{b} \pm 7.99$	$241.66^{b} \pm 9.43$	$286.81^{b} \pm 17.24$
	14 dpiv (12 hpbv)	$53.1^{b} \pm 5.1$	$515.2^{c} \pm 9.78$	$629.17^{c} \pm 56.83$	$450.0^{bc} \pm 31.85$	$10.28^{a} \pm 1.47$	$61.61^{c} \pm 0.69$	$68.19^{b} \pm 2.40$	$64.03^{c} \pm 2.73$	$37.55^{a} \pm 2.89$	$347.16^{c} \pm 9.33$	$412.16^{d} \pm 5.72$	$335.56^{c} \pm 3.57$
	21 dpiv	$54.1^{b} \pm 1.6$	$581.1^{c} \pm 3.22$	$597.22^{c} \pm 10.23$	$490.21^{c} \pm 11.79$	$13.57^{ab} \pm 1.29$	$104.81^{d} \pm 2.86$	$141.13^{c} \pm 10.36$	$490.21^{d} \pm 5.76$	$34.32^{a} \pm 4.91$	$421.96^{d} \pm 3.43$	$386.42^{c} \pm 1.99$	$417.86^{d} \pm 17.69$
iNOS	Zero-day (12 hpiv)	$40.1^{a} \pm 4.1$	$224.9^{b} \pm 9.59$	$63.52^{a} \pm 2.67$	$206.76^{b} \pm 24.85$	$40.05^{a} \pm 3.82$	$128.47^{b} \pm 8.11$	$85.54^{a} \pm 2.59$	$135.91^{c} \pm 7.27$	$50.64^{a} \pm 2.13$	$153.19^{b} \pm 5.67$	$58.11^{a} \pm 2.15$	$130.11^{b} \pm 9.94$
	7 dpiv	$47.7^{a} \pm 5.3$	$57.4^{a} \pm 7.16$	$186.36^{b} \pm 14.27$	$45.72^{a} \pm 5.53$	$49.16^{a} \pm 4.78$	$62.73^{a} \pm 5.29$	$156.04^{c} \pm 6.09$	$76.18^{a} \pm 0.91$	$58.28^{b} \pm 1.71$	$84.87^{a} \pm 1.44$	$212.79^{c} \pm 9.38$	$64.47^{a} \pm 4.37$
	14 dpiv (12 hpbv)	$48.2^{a} \pm 6.7$	$308.3^{c} \pm 20.39$	$360.43^{c} \pm 5.10$	$280.85^{c} \pm 11.99$	$48.78^{a} \pm 4.84$	$167.46^{c} \pm 2.54$	$212.28^{d} \pm 4.26$	$204.96^{d} \pm 1.26$	$67.14^{c} \pm 2.51$	$267.17^{d} \pm 6.31$	$277.47^{d} \pm 8.36$	$268.30^{d} \pm 1.94$
	21 dpiv	$37.4^{a} \pm 1.6$	$61.9^{a} \pm 3.68$	$65.73^{a} \pm 5.93$	$50.19^{a} \pm 5.40$	$47.45^{a} \pm 1.73$	$128.47^{b} \pm 7.56$	$134.56^{b} \pm 7.56$	$113.64^{b} \pm 3.57$	$64.83^{c} \pm 1.79$	$191.26^{c} \pm 11.40$	$187.32^{b} \pm 9.49$	$164.08^{c} \pm 1.23$
TLR22	Zero-day (12 hpiv)	$1852.1^{a} \pm 48.4$	$2006.1^{a} \pm 85.3$	$1999.8^{a} \pm 25.1$	$2542.7^{b} \pm 194.0$	$3867.8^{a} \pm 233.3$	$4173.3^{b} \pm 108.7$	$4060.1^{a} \pm 113.3$	$4470.2^{c} \pm 101.4$	$596.24^{b} \pm 12.42$	$609.62^{a} \pm 26.42$	$595.82^{a} \pm 19.65$	$620.01^{a} \pm 8.63$
	7 dpiv	$2080.2^{b} \pm 87.4$	$2140.8^{ab} \pm 12.2$	$3200.9^{c} \pm 46.1$	$2132.1^{a} \pm 17.7$	$4053.1^{a} \pm 9.7$	$4675.2^{a} \pm 217.5$	$4558.6^{c} \pm 43.3$	$4216.2^{a} \pm 101.4$	$568.47^{ab} \pm 21.14$	$593.57^{a} \pm 11.35$	$636.67^{b} \pm 7.64$	$604.23^{a} \pm 15.11$
	14 dpiv (12 hpbv)	$2051.2^{b} \pm 11.1$	$3743.4^{c} \pm 81.2$	$4667.4^{d} \pm 198.0$	$3522.6^{c} \pm 115.2$	$4408.0^{a} \pm 138.7$	$5087.1^{c} \pm 202.7$	$5934.3^{d} \pm 309.0$	$5572.4^{d} \pm 30.7$	$533.12^{a} \pm 3.69$	$722.47^{b} \pm 11.48$	$753.89^{c} \pm 12.04$	$761.33^{c} \pm 4.78$
	21 dpiv	$2058.7^{b} \pm 26.6$	$2311.3^{b} \pm 22.3$	$2380.2^{b} \pm 26.6$	$2403.2^{ab} \pm 84.9$	$4286.7^{a} \pm 17.4$	$5024.4^{b} \pm 124.2$	$5435.4^{b} \pm 47.9$	$4750.4^{b} \pm 83.1$	$571.45^{ab} \pm 18.86$	$689.14^{b} \pm 15.17$	$723.72^{c} \pm 11.07$	$663.5^{b} \pm 3.48$
NOD1	Zero-day (12 hpiv)	$145.3^{a} \pm 11.9$	$379.6^{c} \pm 2.11$	$145.23^{a} \pm 7.57$	$356.85^{c} \pm 11.13$	$222.99^{a} \pm 3.21$	$381.78^{b} \pm 14.0$	$234.02^{a} \pm 8.0$	$344.45^{b} \pm 13.64$	$186.83^{a} \pm 3.53$	$323.31^{b} \pm 6.55$	$190.76^{a} \pm 5.98$	$280.89^{a} \pm 14.01$
	7 dpiv	$157.9^{a} \pm 1.3$	$185.3^{a} \pm 4.07$	$269.81^{b} \pm 5.80$	$180.73^{a} \pm 7.95$	$244.57^{a} \pm 15.76$	$292.15^{a} \pm 1.22$	$456.2^{c} \pm 9.83$	$276.35^{a} \pm 9.18$	$182.67^{a} \pm 8.02$	$237.99^{a} \pm 8.13$	$351.55^{b} \pm 6.27$	$275.32^{a} \pm 8.53$
	14 dpiv (12 hpbv)	$158.4^{a} \pm 7.9$	$388.0^{c} \pm 5.36$	$414.16^{c} \pm 6.64$	$362.37^{c} \pm 9.76$	$247.79^{a} \pm 12.73$	$446.9^{c} \pm 2.60$	$522.97^{d} \pm 5.58$	$453.87^{c} \pm 9.07$	$173.41^{a} \pm 1.77$	$404.36^{c} \pm 8.58$	$499.41^{d} \pm 10.2$	$427.7^{c} \pm 1.28$
	21 dpiv	$154.2^{a} \pm 1.6$	$237.5^{b} \pm 9.69$	$261.59^{b} \pm 11.68$	$253.65^{b} \pm 3.06$	$223.07^{a} \pm 5.66$	$312.54^{a} \pm 14.01$	$410.21^{b} \pm 6.91$	$317.2^{b} \pm 6.22$	$174.83^{a} \pm 5.22$	$341.83^{b} \pm 7.26$	$405.3^{c} \pm 5.43$	$370.12^{b} \pm 6.88$
IL-1β	Zero-day (12 hpiv)	$702.6^{ab} \pm 15.7$	$1337.1^{b} \pm 47.8$	$687.58^{a} \pm 26.3$	$1484.9^{b} \pm 105.9$	$482.27^{a} \pm 27.93$	$527.31^{b} \pm 3.98$	$483.33^{a} \pm 17.94$	$575.67^{b} \pm 4.33$	$729.01^{a} \pm 5.64$	$1007.0^{a} \pm 8.06$	$764.54^{a} \pm 4.77$	$1209.58^{a} \pm 21.4$
	7 dpiv	$659.6^{a} \pm 8.9$	$957.8^{a} \pm 32.4$	$1338.5^{c} \pm 38.9$	$1004.5^{a} \pm 13.9$	$456.54^{a} \pm 10.07$	$470.23^{a} \pm 6.71$	$598.09^{b} \pm 14.46$	$473.15^{a} \pm 12.78$	$751.94^{ab} \pm 13.14$	$914.2^{a} \pm 25.09$	$1178.62^{b} \pm 25.48$	$1228.85^{a} \pm 52.6$
	14 dpiv (12 hpbv)	$713.5^{b} \pm 23.1$	$3974.1^{c} \pm 43.6$	$3764.7^{d} \pm 37.9$	$3833.2^{c} \pm 76.2$	$496.41^{a} \pm 19.78$	$672.4^{c} \pm 11.29$	$655.72^{c} \pm 13.44$	$627.24^{c} \pm 7.99$	$771.45^{b} \pm 13.71$	$3337.13^{c} \pm 61.19$	$2431.17^{c} \pm 36.25$	$3648.29^{c} \pm 41.3$
	21 dpiv	$691.1^{ab} \pm 9.6$	$906.2^{a} \pm 24.2$	$1232.1^{b} \pm 33.7$	$1090.4^{a} \pm 41.9$	$477.27^{a} \pm 9.28$	$504.11^{ab} \pm 20.52$	$577.88^{b} \pm 22.82$	$589.05^{b} \pm 20.08$	$746.57^{ab} \pm 14.08$	$1792.25^{b} \pm 21.98$	$3079.85^{d} \pm 76.49$	$2696.56^{b} \pm 27.0$

A one-way ANOVA ($p < 0.05$) and Duncan's multiple range test (Superscript lower case letters denote homogenous subsets at different sampling time within the group) was used to determine statistical significance. (hpiv/dpiv – h/d post initial vaccination; hpbv – h post booster vaccination).

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3. Results and discussions

3.1. Positive conjugation of DNA vaccine (pGPD+IFN) with chitosan NPs

In the present study, the DNA vaccine (pGPD + IFN) was conjugated with chitosan NPs by complex coacervation process which involves electrostatic interactions between the free positively charged amino group of chitosan and the negatively charged phosphate group of DNA [13]. Generally for better efficiency of nanoconjugated vaccines small sized NPs (diameters < 100 nm size) are preferred as it assists in better cellular entry and wider tissue distribution [26]. However, the size of DNA conjugated CNPs ranging between 100 nm and 500 nm is also accepted especially in the area of drug delivery systems wherein sufficient amount of drug is needed to be loaded onto the particles in an efficient manner [27,28]. In the present study, the mean particle size of the blank CNPs and DNA conjugated CNPs were measured as 115.9 nm and 306.3 nm, respectively (Fig. 1A). This NPs size falls within the reported size range which could possibly help in the easy delivery of the DNA vaccine (pGPD + IFN) to the host immune cells.

Apart from the size of the particle, the zeta potential or the charge of the particle and the complexation efficiency with pDNA are very important attributes for efficient gene delivery. The NPs having a higher zeta potential value showed better stability than those with a lesser zeta potential [29]. In our study, the zeta potential of blank CNPs and DNA conjugated CNPs were found to be +24.7 mV and +18.0 mV, respectively (Fig. 1B), which is well supported by the results of the previous study by Rojanarata et al. [30] wherein they reported that surface charge of the CNPs prepared with complex coacervation method was slightly positive, with a zeta potential of +9 mV to +18 mV.

Further, the migration of naked plasmid and retardation of conjugated product in the well, as revealed by the gel retardation test, confirmed positive conjugation pGPD + IFN with CNPs. Besides efficient conjugation the efficacy of the DNA vaccine depends on the stability of pDNA *in vivo*. In the present study, the stability test showed that DNase enzymes failed to degrade the pDNA after conjugation ensuring stability of the CNPs-pGPD + IFN construct in hostile environmental conditions like temperature, pH and nuclease activity (in gut for oral vaccine and in water for immersion vaccine) which might help in sustainable delivery of the gene into the host tissues for better expression of the antigenic DNA (Figures in Supplementary file 3).

3.2. Persistence study

In order to investigate how long the DNA vaccine (pGPD + IFN) is able to release the recombinant GAPDH (as antigen) inside the host after immunization, PCR amplification for GAPDH gene (996 bp) was done with DNA extracted from specific tissues collected from different experimental groups (as mentioned earlier) at 14 day and 35 day post initial immunization. The PCR results (Supplementary file 3) displayed that the immunized fish (all 3 immunized groups) contains gene transcripts for GAPDH gene not only at 14 day post initial immunization but also at 35 day post vaccination. Previous studies also reported that DNA constructs are capable of persistent expression of immunogenic proteins in host tissues and protective response up to 50 days or more [31]. Thus from our results it can be ascertained that there was sustained expression of GAPDH protein from the recombinant plasmid after administration in the *L. rohita* (independent of immunization strategy) which in turn helps in inducing protective immune responses in the host to effectively counter *E. tarda* infection.

3.3. Challenge study and relative percentage survival (RPS) analysis

After challenge with the bacterium, the moribund fishes across various experimental groups viz. pGPD + IFN (injection), CNPs-pGPD + IFN (oral), CNPs-pGPD + IFN (immersion) and control groups showed typical signs of acute septicaemia with ascitic dropsy, external lesions, erosion of skin, haemorrhages and congested vent starting at 36–48 h post challenge (hpc). Haemorrhages were seen in the internal organs including liver, kidney and spleen. Clinical signs and histopathological manifestation in kidney and liver tissue sections 48 hpc from all the experimental groups were attached in Supplementary file 3. The survived fish demonstrated no gross lesion or clinical signs typical of edwardsiellosis, however, few fishes showed mild congestion at ventral parts of body during peak infection stage which subsided in the later phase of infection. Mortality was observed first time at 2 day post challenge (dpc) and continued up to 11 dpc after which no mortality was observed. From the challenge study (Fig. 2), it was revealed that, the CNPs-pGPD + IFN (oral) immunized group recorded highest RPS of 81.82%, followed by CNPs-pGPD + IFN (immersion) immunized group (72.73% RPS) while the pGPD + IFN (injection) vaccinated group showed 63.62% when compared with the cumulative mortality percentage in the control group (55%). The cause of mortality was confirmed by re-isolating the bacteria from kidney of freshly dead or moribund fish. The RPS data for pGPD + IFN (injection) group was similar to our previous study [3] with injection of naked pGPD + IFN vaccine (63.16% RPS) but interestingly the CNPs conjugated vaccine showed much higher RPS (both in oral and immersion group). Similar RPS (>80%) were previously recorded in orally immunized fish where NPs were used as vaccine carrier [32–35] suggesting that CNPs conjugated DNA vaccine can be delivered effectively by oral or immersion route and be a promising alternative for injection vaccine. In addition, the mortality results also highlighted how most of the control fish succumbed to pathogen-mediated lethality while fish in the vaccinated groups evade the bacterial infection elucidating the protective efficacy of constructed DNA vaccine (pGPD + IFN) against *E. tarda*.

3.4. Evaluation of non-specific immune responses

Besides the RPS analysis, assessments of innate immune responses are important aspects in evaluating vaccine efficacy. The respiratory burst activity (NBT) and myeloperoxidase activity (MPO) are oxygen-dependent reactions which are commonly used to evaluate the defense ability of the host against pathogens [36,37]. In the present study, the immunized groups showed significantly ($p < 0.05$) higher NBT activity (Fig. 3A) and MPO activity (Fig. 3B) post vaccination (both initial and booster dose) when compared with the control group. Moreover, the CNPs-pGPD + IFN (oral and immersion) immunized groups showed relatively higher activity than the pGPD + IFN (injection) group. At 48 hpc, NBT and MPO activities increased significantly ($p < 0.05$) irrespective of groups but the CNPs-pGPD + IFN (immersion) group displayed highest activities in both the cases. Similar enhancement of NBT and MPO activities was also demonstrated in fish immunized by DNA vaccines [38,39] indicating possible neutrophils activation in the host after countering foreign antigen (in vaccine) or whole bacterium (after challenge) which ultimately helped in the clearance of the bacteria.

Serum lysozyme activity is an important non-specific humoral defense component of white blood cells, which can decompose cells by hydrolyzing peptidoglycan in bacterial cell wall [40]. In the current study, there was significant ($p < 0.05$) enhancement of lysozyme activity (Fig. 3C) in the immunized groups when compared with the control group at all time points. Furthermore, the CNPs-pGPD + IFN (immersion) group showed higher lysozyme

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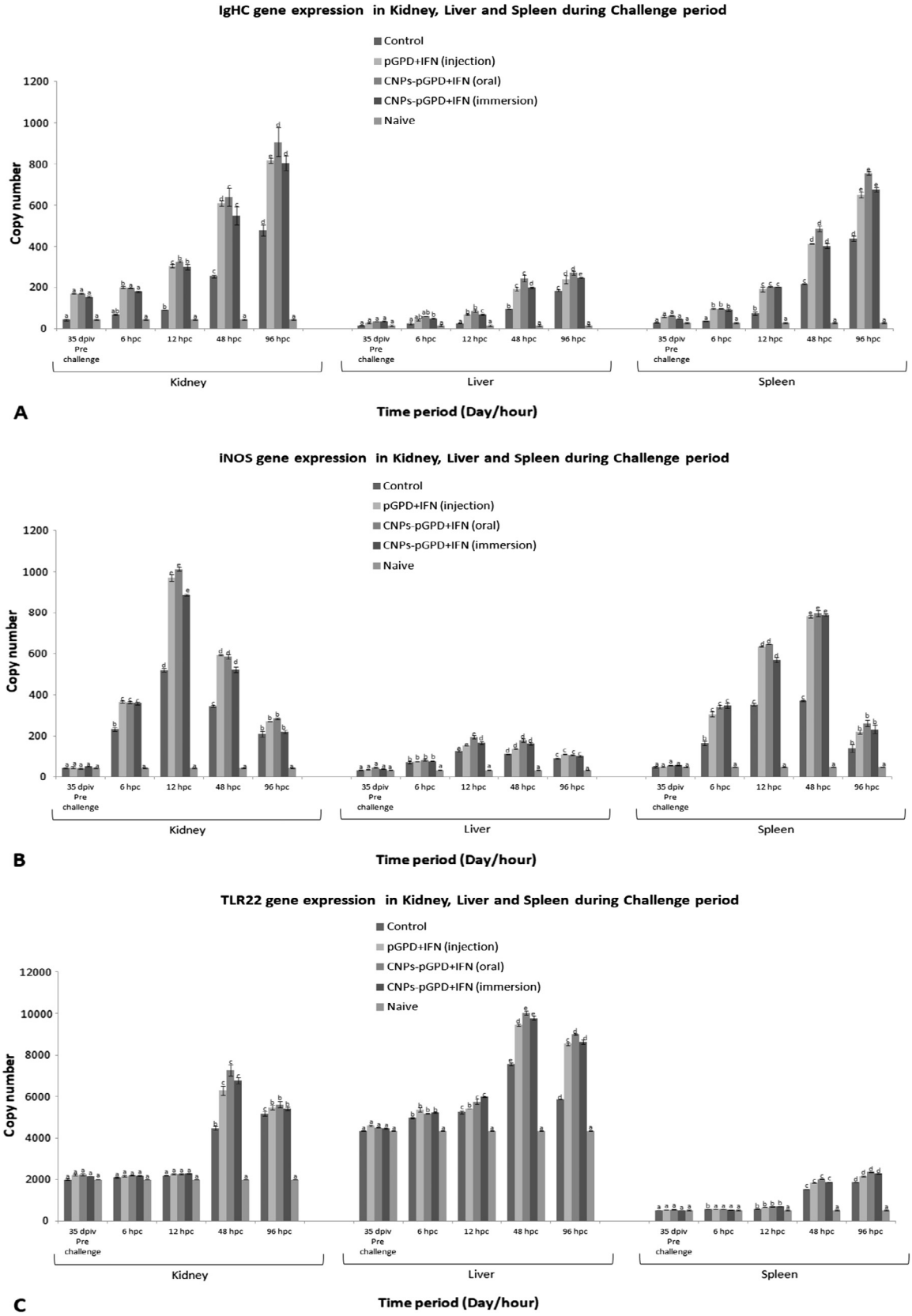

Fig. 5. Absolute quantification of IgHC (A), iNOS (B), TLR22 (C), NOD1 (D) and IL-1β (E) genes in kidney, liver and spleen of *Labeo rohita* of different experimental group viz. pGPD + IFN (injection), CNPs-pGPD + IFN (oral), CNPs-pGPD + IFN (immersion), Control and Naive group at different time intervals during challenge (35 dpiv-pre challenge and post challenge) with *E. tarda* (8.7×10^4 CFU/fish). The mean values (n = 3) of each gene transcript in the kidney, liver and spleen tissues were plotted with standard error as a function time at pre and post challenge. A one-way ANOVA ($p < 0.05$) and Duncan's multiple range test (homogenous subsets indicated by alphabets) was used to determine statistical significance of the tissue specific IgHC, iNOS, TLR22, NOD1 and IL-1β gene expression at various time points. (hpiv/dpiv – h/d post initial vaccination; hpbv – h post booster vaccination).

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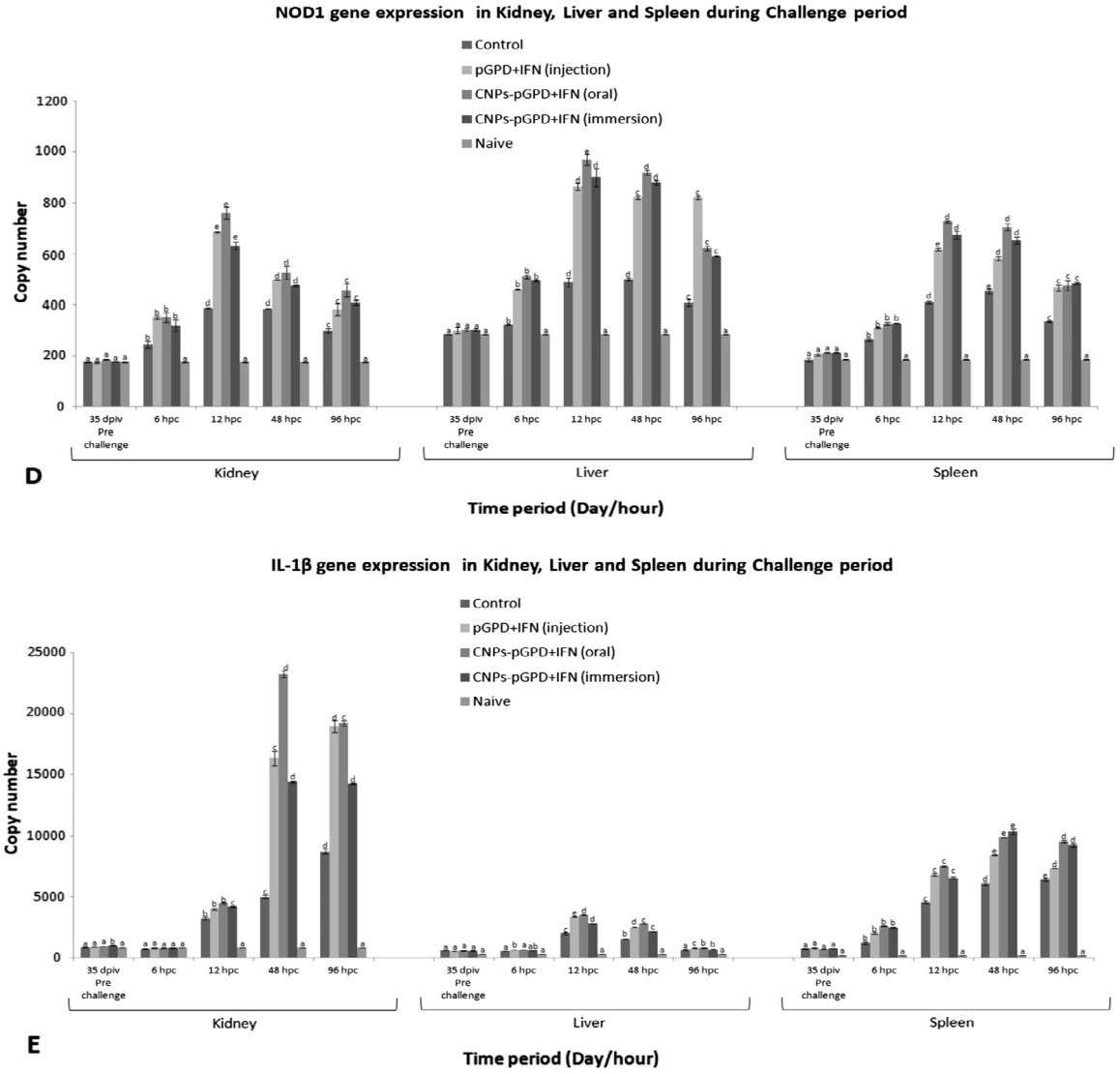

Fig. 5 (*continued*)

activity than other two immunized group during immunization period as well as 48 hpc. Several other studies also reported increased lysozyme activity in fish immunized by nanovaccines [39,41] demonstrating the correlation of lysozyme activity with the efficacy of immunization strategy in establishing an innate protective mechanism in the host against pathogens. Thus, from the non-specific immune responses it can be assumed that nanoconjugation of the DNA vaccine positively affects the release of recombinant antigens in the host which in turn stimulates the host immune cells in orchestrating a combined defense mechanism against bacterial challenge.

3.5. Specific antibody quantification

Apart from non-specific immune assays, a competitive ELISA (Fig. 4) was employed for the detection of fish-anti-GAPDH antibody. The present results significantly ($p < 0.05$) demonstrated higher immune response in the immunized groups than the control group both during pre and post challenge period. The increasing trend for antibody titre from 7 day post immunization to 21 day post immunization (after booster dose) coupled with minimal level of titre recorded after initial immunization signifies gradual production of IgM in response to the DNA vaccine. Although there was little difference in the titre level between the immunized group during vaccination period but interestingly during the peak infection phase (post challenge) the CNPs-pGPD + IFN (oral) group showed higher antibody titre followed by CNPs-pGPD + IFN (immersion) and pGPD + IFN (injection) groups. This can be correlated with the mortality pattern in different immunized groups where higher specific antibody level in the respective group apparently helps to overcome the bacterial pathogenicity. Furthermore, the results displayed enhanced antibody levels in the CNPs conjugated DNA than naked DNA vaccine. Similar findings of increased antibody titre in response to nanodelivered DNA vaccines were recorded previously [14,42,43]. These results possibly explain sustained release of the antigens from nanodelivered vaccine which results into enhanced production of specific antibody creating an anti-microbial environment in the host. However, the exact reason behind the higher antibody titre in the nano-vaccinated group is not well understood suggesting a further detailed study.

3.6. Expression kinetics of immune-related genes

In addition to the non-specific and specific immune responses, we have also studied the expression kinetics of five different classes of immune-related genes (IgHC, iNOS, TLR 22, NOD 1 and IL-1β) in three immunologically important tissues (kidney, liver

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and spleen) at post immunization (Table 1) as well as post challenge period (Fig. 5).

Consistent with the production of specific antibodies during the immunization process, IgHC (IgM heavy chain) gene transcript was upregulated in the immunized groups from 7 day post immunization with highest expression recorded in kidney of CN Ps-pGPD + IFN (oral) group at 14 day post immunization followed by pGPD + IFN (injection) and CNPs-pGPD + IFN (immersion) groups, after which the expression remained stable at 21 day post immunization. However, in the spleen pGPD + IFN (injection) and CNPs-pGPD + IFN (immersion) groups showed higher expression of IgHC transcript than in CNPs-pGPD + IFN (oral) group. The expression kinetics can be correlated with the antibody titre as IgHC is an indicator of specific immune responses in teleost fish [44]. Also, highest fold changes of IgHC copies was observed in spleen tissue (irrespective of immunized groups) suggesting enhanced secondary immune response in the spleen. Moreover, during challenge period, IgHC expression in all the tissues increases significantly ($p < 0.05$) from 48 hpc with highest IgHC transcript level recorded in CNPs-pGPD + IFN (oral) group at 96 hpc. The up-regulation of IgHC in the CNPs-pGPD + IFN (oral) group is supported by the previous findings [14,15,41] wherein oral delivery of nanovaccine in fish resulted in high expression of IgHC.

The current study investigated the expression profile of iNOS (Inducible nitric oxide synthase) gene, a multifunctional effector molecule and a immune-regulatory factor important in the defense against various pathogens [25,45]. Although, the iNOS gene transcript increased little (~250 copies in immunized groups against ~50 copies in control group) during the vaccination period in all three tissues, the copy number in all challenged groups raised abruptly post challenge with respect to naive fish. Unlike IgHC, kidney showed highest iNOS expression followed by spleen at 12 hpc. The highest expression of iNOS gene in the CNPs-pGPD + IFN (oral) group post challenge might have helped in the production of reactive nitrogen intermediates (RNIs) which may inhibit the pathogen at the initial phase of infection leading to better protective immune response than other experimental groups especially the control group where prolonged mortality was observed.

Furthermore, the study also included the expression kinetics of genes involves in PRR (pattern recognition receptors) pathways, TLR22 (Toll like receptor 22) from TLR pathway and NOD1 (Nucleotide binding and oligomerization domain-1) from NLR pathway. TLR22 which recognizes bacterial RNA plays a distinctive role in innate immune responses in teleost [46]. Significant increase in TLR22 gene transcript was observed in immunized group (in all 3 tissues) only at 14 day post immunization as compared to the control group. However after challenge with bacterium, TLR22 mRNA transcript increased in all the experimental groups (included control group) at 48 hpc. The CNPs-pGPD + IFN (oral) group showed highest expression in kidney and liver at 48 hpc and in spleen at 96 hpc. The delayed upregulation of TLR22 observed after bacterial challenge is in line with previous findings [46,47] where significant upregulation of TLR22 was reported at 48–72 hpi. Like TLRs, NOD1, the member of the NLR family senses a wide range of bacteria and viruses or their products, and plays a key role in inducing innate immunity. The results showed immediate upregulation of NOD1 gene transcript in pGPD + IFN (injection) and CNPs-pGPD + IFN (immersion) groups post vaccination (both after initial and booster dose) but later subsided while the CNPs-pGPD + IFN (oral) showed increase in NOD1 transcripts from 7 day post immunization with highest expression on 14 day after vaccination. Moreover, the NOD1 gene transcript (in all 3 tissues) increases significantly at 12 hpc but declined after 48 hpc as compared to the naive fish group. Similar upregulation pattern was noticed in previous studies [48,49] wherein highest NOD1 expression was observed at 12–48 hpc. Thus, the elevated expression of PRR genes in the CN Ps-pGPD + IFN immunized groups post challenge signifies the utility of nanoconjugation in inducing effective immune responses in the host.

The present study also analysed the kinetics of IL-1β (Interleukin-1β), a typical pro-inflammatory cytokine and a major player in immune response in fish [50]. The expression pattern of IL-1β was similar to the NOD1 expression during immunization period. However, after challenge, the IL-1β expression in all the tissues increased significantly from 6 hpc with highest gene transcripts recorded in kidney of CNPs-pGPD + IFN (oral) group and in spleen of CNPs-pGPD + IFN (immersion) group at 48 hpc when compared with the naive group. The upregulation of IL-1β in the CNPs-pGPD + IFN immunized groups at early stages of infection might have helped in combating the disease leading to better host survival.

In conclusion, our study revealed that CNPs-pGPD + IFN is an effective immunization strategy for protection against edwardsiellosis in *L. rohita*. The high RPS values obtained in the present experiment shows that nanoconjugation of pDNA enhanced the vaccine efficacy and offered better protection to fish compared to naked pDNA. In addition, various immunological parameters also demonstrated the positive effects of the nanovaccines for induction of coordinative immune responses in the host against bacterial challenge. Moreover, the differences in immune responses and RPS observed from different groups elucidates that delivery routes has a considerable impact on the vaccine efficacy. Thus, it can be inferred that CNPs conjugated pGPD + IFN could be an efficient immunization strategy against *E. tarda* in *L. rohita*, which can be administered by oral or immersion route (minimum stress delivery methods) in the host with maximum effectiveness.

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Declarations of interest

None.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2018.02.099.

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