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Bacterial DNA pre-stimulation elevates antiviral gene expression in koi against koi ranavirus (KIRV) infection

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Abstract
The present experiment was conducted to investigate the expression of innate immune-related genes TLR9, type I IFN and Mx3 in tissues of kidney and spleen in juvenile koi (*Cyprinus carpio* L) after induction with bacterial DNA (bDNA) stimulation, koi ranavirus (KIRV) infection, and combined administration bDNA followed by the virus. The study revealed that the mRNA expression of TLR9 was continuously upregulated in kidney between 24 h (35 fold) to 96 h (126 fold) in bDNA pretreated fish infected with KIRV. TLR9 was upregulated in spleen from 1.1 fold (24 h) to 19 fold (96 h) when stimulated with bDNA to 4.11 fold (96 h) upon virus administration. Type I IFN gene was upregulated in spleen of koi at 24 h (50 fold) and decreased later till 96 h (1.08 fold) during virus infection. The expression of IFN in both kidney and spleen was down regulated from 24 h to 96 h post infection in both fish treated with bDNA and virus infected fish pre-treated with bDNA. Infection with KIRV induced high Mx3 expression at 24 h in kidney in all three treatments, while it showed increasing trend in spleen with high expression at 96 h especially in virus infected fish pretreated with bDNA. These studies revealed that the mRNA expression profiles of immune-related genes in koi were stimulated by bDNA and KIRV. The study also revealed the immune-stimulatory potency of bDNA to enhance innate immune response similar to CpG ODNs that could be used as adjuvant preparation in viral vaccines for fish

Keywords: Koi ranavirus, gene expression, Immune related genes, TLR9, Type I interferon, Mx-3, bacterial DNA

1. Introduction
Koi is one of the most colourful varieties of common carp, grown by personal hobbyists and also recognized as a cultured ornamental fish species for trade and export. Infection caused by iridoviruses in koi has not been reported so far. However, recently the first occurrence of a new ranavirus infection associated with mortalities was reported from koi in South India [1]. An iridovirus disease outbreak causes several clinical signs mainly associated with death and some time without any clinical signs depending on the infected host species [2]. The family Iridoviridae is further subdivided into five genera, *Iridovirus*, and *Chloriridovirus* that infect the invertebrates while other genera of *Ranavirus*, *Lymphocystivirus*, and *Megalocytivirus* that infect the cold-blooded vertebrates [3]. Particularly genus of *Ranavirus* and *Megalocytivirus* are now recognized as emerging pathogens that mainly affect the fish and amphibians [4]. Reports in the past 30 years indicate that the ranavirus infection causes several clinical signs, shows considerable morbidity and mortality in ectothermic vertebrates including reptiles, amphibians and fish [4, 5]. The first documentation of Santee-Cooper ranavirus-like viral agent of koi ranavirus (KIRV) has been detected and isolated from infected koi in South India [1].
The primary task of recognition of invading microorganisms involves action of different germline-encoded innate immune sensors like pattern recognition receptors (PRRs), which are toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I) and RIG-I-like receptors (RLRs) that target overlapping different classes of pathogens and share functional domains and signal transduction pathways [6-8]. PRRs are rapid in recognizing the conserved microbial structures, molecular motifs (pathogen associated molecular patterns; PAMPs) as well as the immunogenic endogenous molecules released by infected hosts [9-11]. Viral and bacterial infections activate the interferon (IFN) signalling mechanism of innate immunity. The interferons (IFNs) are subdivided into three types including type I IFN (α and β), type II IFN (γ) and type III IFN (λ1, λ2, λ3) that are primarily present in mammals and type I and type II

IFNs are stimulated directly due to virus infection [12], except type III IFNs. Diverse group of interferons stimulated genes (ISG) including dsRNA dependent protein kinase R (PKR), Mx and 2-5 oligo (A) synthetase (2-5 OAS) that are expressed following virus infection inhibit replication of the virus in the host cell through JAK-STAT pathway, which transmits the signal to the nucleus by secreted IFNs [13-16]. The different groups of bacteria and virus pathogens are recognized by several intracellular TLRs such as TLR3, TLR7, TLR8, and TLR9. Among these TLRs, TLR3, TLR7/8, and TLR9 particularly recognize the different classes of nucleic acids like single-stranded RNA (ssRNA), double-stranded RNA (dsRNA) and CpG DNA respectively [17]. TLR7, TLR8, and TLR9 direct adaptor molecule of myeloid differentiation factor 88 (MyD88) which mainly induce the immune responses while TLR3/TLR22 depend on TIR domain-containing adaptor-inducing IFN-β (TRIF) that mediate downstream signalling pathways for the period of both virus infection or PAMPs stimulation [18, 19]. MyD88 and TRIF are generally activated to nuclear factor-kB (NF-kb) and interferon regulatory factors-3/7 (IRF-3/7) [20]. CpG ODNs are now recognized as immunomodulators capable of acts as adjuvant with vaccines. However, as synthetic CpG ODNs are very expensive, in the present research work, an attempt is made to find out the ability of bacterial DNA to act as an immunomodulator in viral vaccines.

2. Materials and methods

2.1. Fish

The koi (*Cyprinus carpio* L) juveniles (average weight 10 g) were obtained from the local ornamental fish farm (Thoothukudi, India) and were maintained in 50 L rectangular glass aquarium tank at a temperature of 29 ± 1 °C. Before starting the challenge study, the fish were acclimatized to laboratory condition for 7 days prior to use in experimental infection and fed a commercial diet twice daily.

2.2. Isolation of bacterial strain

Virulent *Aeromonas hydrophila* strain used for this study was isolated from diseased *Pangasius* sp. *A. hydrophila* was grown in Tryptic soy broth (TSB) and Tryptone soya agar (TSA) at 37 °C. Bacterial suspensions were prepared in 5 ml of sterile TSB broth incubate overnight at 37 °C and 1 ml overnight culture aliquots were inoculated into 100 ml of TSB broth for mass culture incubate in shaking incubator at 37 °C. The density of aliquot of overnight mass culture absorbance at 600 nm (OD600) measured on a double beam UV-V spectrophotometer (Hitachi - Science & Technology, Japan). The DNA was extracted using HIMEDIA kit as per the manufacturer's instructions. The concentration of extracted DNA was measured by UV spectrophotometer at OD260 nm.

2.3. Viral strain and propagation

Indian strain of koi ranavirus (KIRV) isolated from infected koi and characterized in the Department of Fish Pathology and Health Management was used for this study [1]. *Epithelioma papillosum cypini* (EPC) cell line was used for isolation, propagation and infectivity assay of this virus. The cell line was maintained in Leibovitz-15 (L-15) medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 1x antibiotic-antimycotic solutions (Gibco, USA) in 25 cm^2 cell culture flasks (Thermo, Korea) at 27 °C.

2.4. Immune stimulation and challenge of koi fish

During the treatment and challenge period, the fish was sedated with 0.04 g/l benzocaine (Sigma, St. Louis, USA). Koi is divided into four groups (9 fish/group) each in duplicate was used for experimental study of gene expression (Fig 1). First two groups of fish were administrated intramuscularly (i.m) with 50 µl of phosphate buffered saline (PBS). After 24 h PBS injection, one of these group of fish were challenged intraperitoneally (i.p) with 50 µl KIRV ($10^{6.9}$ $TCID_{50}$/ml) as PBS-virus group while the other group of fish was administered with a repeat dose of PBS at the same concentration as control PBS group. The next two groups of fish were administered intramuscularly (i.m) with 50 µl of bacterial DNA at 10µg/fish. After 24 h post-administration of bacterial DNA stimulation, one of these group of fish were challenged intraperitoneally (i.p.) with 50 µl virus ($10^{6.9}$ $TCID_{50}$/ml) as bacterial DNA-virus group while the other group of fish administered with a repeat dose of bacterial DNA at the same concentration as control bacterial DNA group. After 24, 48 and 96 h post-induction with bacterial DNA or PBS, tissue samples such as kidney and spleen were collected from both control and treatment groups (3fish/time point) and stored in RNAlater (Qiagen) at – 80 °C until RNA isolation.

2.5. Total RNA extraction, cDNA synthesis, and gene sequencing

The level of expression of immune genes such as TLR9, IFN, and Mx3 were evaluated in the experimental fish from test groups by quantitative real-time RT-PCR method. Total RNA was extracted from tissues of the kidney and spleen from experimental fish in each group at 24, 48 and 96 h after injection by using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. Finally the extracted RNA was eluted in 100 µl of DEPC treated RNase-free distilled water. The 1 µg of total extracted RNA from each sample was used for reverse transcriptase using the cDNA Synthesis Kit (Thermo Fisher Scientific, USA). The amplification of different immune related-genes and reference gene β-actin (M24113) that degenerated primers were designed based on the conserved region of following reported genes of common carp (TLR9, GU809229; Type I interferon, GQ168344; Mx3, EF635410) sequences from Genbank. Details of primers are given in Table 1. The reaction mixture contained 10 µl of extracted RNA with 10 µl of cDNA synthesis mixture was prepared as per the manufacturer's instruction. The reaction mixture was incubated at 25 °C/10 min; 37 °C/160 min and 85 °C/5 min.

2.6. Quantitative real-time reverse-transcriptase PCR for immune genes

The Real-time PCR amplification was performed in Step-One plus Real-Time PCR Detection system (ABI, Invitrogen, Life sciences, USA) by using Power SYBR® Green PCR Master mix (Thermo Fisher Scientific, USA). The 2 µl of aliquot of cDNA was analyzed by qRT-PCR in the 25 µl reaction mixture containing 12.5 µl of 2x power SYBR green (Thermo, USA), 2 µl of each forward and reverse primer, and 6.5 µl of nuclease free-water. The standard cycling conditions were as follows: 95 °C/10 min and 40 cycles of 95 °C/15 s, 54 °C/1 min for (TLR9, Mx3 and β-actin), 60 °C/1 min (type I IFN), and 72 °C/1 min. All experiments were analyzed in triplicates. The mRNA expression levels of immune genes were calculated by using the comparative threshold cycle

method ($2^{-\Delta\Delta CT}$) with β-actin gene as an internal control.

3. Results

For this study, juvenile koi fish were stimulated with bacterial DNA and KIRV infection for elevation of antiviral genes. The experimental fish groups were examined for the expression immune-related genes like TLR9, type I IFN, and Mx3 in tissues of the kidney and spleen in koi at 24, 48 and 96 h post-challenge. The following results showed that treatment with bDNA stimulation and KIRV infection modulates the immune response in fish.

3.1. Expression of immune genes of TLR9, type I IFN and Mx3 in kidney tissue of koi

The expression of TLR9 gene in kidney of koi when the fish are infected with KIRV and treated with bDNA alone showed 146 and 351 fold inductions respectively, while virus infected fish pre-treated with bDNA showed only 35 fold induction at 24 h post-induction (Fig. 2). At 48 h post induction, TLR9 expression was reduced, when fish administrated with KIRV alone (1.23-fold) and virus infected fish pre-treated with bDNA showed 2.15-fold inductions. On the other hand, fish are treated with bDNA alone showed 3.88 fold inductions. In 96 h post-induction TLR9 gene expression was high on virus infected fish pretreated with bDNA showed 126 fold induction, compared to the infected fish with KIRV alone 2.7 fold induction. At the same time point, treatment with bDNA alone showed 3.25 fold induction.

The expression of type I IFN gene in kidney of koi was 2513 and 2366 fold respectively in the virus infected fish pre-stimulated with bDNA as well as induced with bDNA alone compared to when fish are infected with KIRV alone (1744 fold induction) at 24 h post-induction (Fig.3). At 48 h post-induction, IFN gene expression showed 100 fold induction in the fish treated with bDNA alone, while expression reached to normal level in other groups of fishes that were infected with KIRV alone and KIRV infected fish pretreated with bDNA. IFN gene was expressed 306 fold induction in fish infected with KIRV alone, while it was 3.6 fold following treatment of fish with bDNA followed by KIRV infection at 96 h post-induction. Treatment of fish with bDNA alone showed 384 fold induction.

Expression of Mx3 gene in kidney of koi stimulated with bDNA alone showed 138 fold induction at 24 h. At the same time, expression of Mx showed high (115 fold) induction in virus infected fish pre-treated with bDNA, compared to the KIRV infection alone which showed 90.44 fold inductions (Fig. 4). At 48 h post-induction, the expression of Mx3 gene in fish treated with bDNA alone showed 7.28 fold induction. Expression of Mx3 gene in fish treated with bDNA alone showed 4.65 fold induction while expression was.35 and 2.24 fold induction at 96 h in virus infected fish pretreated with bDNA and fish infected with KIRV respectively

3.2. Expression of immune genes TLR9, IFN and Mx3 in spleen tissue of koi

TLR9 gene was not up-regulated in the different treatment groups at 24 h (Fig.5). Fish treated with bDNA alone showed 5.22 fold induction at 48 h followed by KIRV infected fish pretreated with bDNA which showed 2.27 fold induction. The expression of the gene showed 19 fold inductions in fish stimulated with bDNA alone while 4.11 fold inductions found on fish infected with KIRV at 96 h.

The expression of type I IFN showed 29 fold induction in KIRV infected fish pretreated with bDNA at 24 h (Fig.6). At the same time, fish infected with virus showed 50 fold induction. At 48 h post-induction, fish infected with KIRV showed 3.41 fold induction, while gene was down-regulated in the other two groups of fish treated with bDNA alone and KIRV infected fish treated with bDNA. At 96 h post-induction, bDNA injected fish infected with KIRV showed 12 fold induction.

No upregulation of the gene Mx3 was observed in any treatment group at 24 h (Fig.7). At 48 h post-induction, high upregulation was found in bDNA injected fish infected with virus showed 11.27 fold induction. At the same time, fish treated with bDNA alone showed 3.07 fold induction and fish infected with KIRV showed1.82 fold induction. At 96 h post-induction, fish infected with virus showed 34.36 fold induction. At the same time point, 19 fold induction was observed in virus infected fish pre-treated with bDNA, while fish treated with bDNA alone showed 10.90 fold inductions.

4. Discussion

4.1. Expression of the TLR9 in kidney and spleen tissue of koi after stimulation

The unmethylated CpG motifs of bacterial DNA are recognized by the innate immune system through the signal of infectious threat that triggers the innate immune defence mechanisms of the host animal. The present study demonstrated for the first time the production of innate immune-related genes such as toll-like receptors 9 (TLR9), type I interferon (type I IFN) and Mx3 (antiviral genes) in juvenile koi fish were induced by the use of bacterial DNA, which acts as an immunomodulator. The expression of the gene TLR9 in kidney and spleen tissues of koi was found stimulated with bDNA and KIRV injection at different time points of post induction as analyzed by qRT-PCR. The present experiment result showed that the expression of TLR9 in kidney tissue of koi highest level of up-regulation at 24 h (146 fold) and later down-regulation was recorded at 96 h (2.7 fold) post infection in fish infected with virus. Whereas continuous up-regulation of the gene in the kidney on KIRV infected fish pre-treated with bDNA ranging from 24 h (35 fold) to 96 h (126 fold).

The mRNA expression of the gene TLR9 in spleen tissue of koi continuously up-regulated from 24 h (0.60- fold) to 96 h (4.11-fold) in fish infected with KIRV, while virus infected fish pre-treated with bDNA showed up-regulation at 48 h (2.27-fold) and later down-regulation at 96 h (0.45-fold). Our result compared well with that of an earlier report where TLR expression was down-regulated in response to CpG-B compared to other class of CpG-A and CpG-C at 2nd day, however slightly up-regulated at 5th day in Atlantic salmon with TLR9 ligands [21]. CpG ODN are subdivided into A, B and C which is characterized by their structural differences and stimulation of different immune responses [22]. In fish, A and B ODNs, particularly the latter which includes ODN 2006, ODN1668 and ODN1826 have immunostimulatory properties [23-25].

In current study, expression of the gene TLR9 in the kidney of koi treated with bDNA alone showed up-regulation at 24 h (351-fold) and later down-regulation ranging from 48 h (3.89-fold) to 96 h (3.24-fold). The expression of TLR9 gene in spleen tissue was up-regulated in fish pretreated with bDNA alone at 96 h (19-fold). The up-regulation of the immune-related gene of TLR9 induced by CpG ODN was reported from several fish species like Atlantic salmon, Japanese

flounder and sea bream [26-29]. Experimental study of megalocytivirus in Japanese flounder pre-treated with different classes of CpG ODN like C3, C5, and C7, showed that viral load was significantly reduced in fish tissues during the experimental duration, suggesting that ODNs of C3, C5, and C7 have antiviral properties [30]. The bacterial DNA contains unmethylated CpG motifs, which are recognized by vertebrate innate immune defence system as well as induced the non-specific immune responses [31]. The synthetic CpG oligodeoxynucleotides (ODNs) up-regulated several immune genes related to TLR9 signalling pathway, especially IRFs, IL-1b, TLR9, type I IFN, and Mx in tissues of such as kidney, spleen, liver in several fish species including Atlantic salmon, gilthead sea bream and Japanese flounder [21, 27, 30, 32]. Present study therefore indicated that the bDNA is almost equivalent to CPG motifs capable of stimulating the immune genes.

4.2. Expression of type I IFN in kidney and spleen tissue of koi after stimulation

The mRNA expression of the type I IFN has been reported from several fish species including Atlantic salmon, channel catfish, fugu, goldfish and zebrafish [33-37]. The expression of type I IFN in kidney tissue of koi up-regulated at 24 h in all the treatments and the fish pre-treated with bDNA followed by KIRV infection showed highest expression profile (2513-fold). In this study, highest upregulation of type I IFN was found in kidney tissue of koi at 24 h and later it was declined. This result corresponds to previous findings reported in Atlantic salmon [32, 34].

The expression of type I IFN in the spleen of koi was up-regulated at 24 h (50- fold) in KIRV infected fish. At the same time point, gene expression was continuously down-regulated ranging from 48 h (3.41-fold) to 96 h (1.08-fold). The level of type I IFN gene expression in spleen tissue of koi when fish were pre-treated with bDNA followed by KIRV infection was up-regulated at 24 h (29-fold) and decreased later at 48 h and slightly increased at 96 h (12.02-fold). The expression of type I IFN gene in spleen of koi infected with KIRV alone showed steady down-regulation from 24 h to 96 h. These results are in agreement with the previous reports of type I IFN observed in all experimental groups at 1 and 2 day post-infection with viral haemorrhagic septicaemia virus (VHSV) in olive flounder (*Paralichthys olivaceus*) [38]. These results also agree to an earlier study which provided strong confirmation for the existence of interferon (IFN) as an antiviral innate immune defence mechanism in fish [37]. The results showed that the expression of type I IFN gene in kidney was down-regulated from 24 h (2366-fold) to 96 h (383-fold) in fish with bDNA stimulation similar to KIRV infected fish treated with bDNA was down-regulated from 24 h (2513-fold) to 96 h (3.57-fold).

Current study indicated that the type I IFN gene expression was mediated by viral infection and also bacterial stimulation in tissues of kidney and spleen. The stimulation of gene expression was high in viral infection compared to bacterial stimulation. In an earlier report the type I IFN gene was not detected in the uninfected common carp but was elevated by imiquimod and poly I: C stimulation [39]. Type I IFN was found expressed constitutively in the uninfected fish as well as conspicuously up-regulated in virus infected fish such as rainbow trout [40] and olive flounder [41].

4.3. Expression patterns of Mx3 in kidney and spleen tissue of koi after stimulation

The level of Mx3 gene expression in kidney tissue of koi was up-regulated at 24 h (90-fold) in KIRV infected fish and declined at 48 h (1.67-fold) and slightly increased at 96 h (2.24-fold), while compared to fish treated with bDNA followed by virus infection where it was up-regulated at 24 h (115-fold) and remained at decreased level at 48 and 96 h (3.35-fold).

The mRNA expression of Mx3 gene in spleen tissue of koi was up-regulated from 48 h (1.82-fold) to 96 h (34-fold) in KIRV infected fish. Similar nature of expression prevailed in fish treated with bDNA at 48 h (11.28-fold) and 96 h (20-fold). The mRNA expression of Mx3 gene was up-regulated in kidney and spleen at 24 and 96 h after stimulation with bDNA and virus infection showed that the treatment of fish with bDNA could induce the antiviral activity and decrease the viral load in the host animal. This observations in the current study is similar to the earlier reports of mRNA expression of Mx gene in Japanese flounder infected with the HRV (Hirame rhabdovirus) which was up-regulated at 48 h and peaked at 72 h [42] and other studies that revealed that the dual infection of infectious pancreatic necrosis virus (IPNV)-infectious haematopoietic necrosis virus (IHNV) up-regulated Mx gene expression, which peaked at 2nd day post-induction and remained high until 7th day [43]. The mRNA expression of Mx gene in olive flounder was significantly elevated at 1 day post induction and remained elevated until 7 day post induction after VHSV infection [38]. The expression of Mx gene in Atlantic salmon was detected at 2nd day post induction and later increased at 4th day post induction and remained undetected at 7th day post induction due to poly IC induction [44]. Expression of Mx gene stimulated by viral infection has been reported in different fish species by several researchers [41, 45-47] which also involved in the elimination of the viral particles from the host [48]. The findings indicated that Mx gene is stimulated by the presence of IFN transcripts occurring in animals [49 -51]. The effective immunostimulatory property of bacterial DNA in koi reported in our present study on the immune related genes like TLR9, type I IFN, and Mx3 aids the main theme that the bacterial DNA can act as an immunemodulator for fish viral vaccines. In the present study on innate immune-related gene response of juvenile koi, we report for the first time the role of bacterial DNA in inducing innate immune response and induction of antiviral activity indicated by Mx gene expression as well as its adjuvant potential that could be effective in the control of ranavirus infection in koi.

Table 1: Primers used in this study

Gene	Primer	Primers Sequences 5'-3'	Product size (bp)	Accession No	Reference
Type I IFN	CCRINTFW	TGCATATGGCTCGGCCAATA	167	GQ168344	Kitao *et al.* [39]
	CCRINTRE	GTCAAGACAAGAAACCTCACC			
TLR9	CCRTL9FW	TAAGGTGGGTGGGGCTTTC	88	GU809229	Adamek *et al.* [51]
	CCRTL9RE	CGGTAGTTGTTTGGTATGGCTTC			
Mx3	CCRMxRFW	TTGAAGGAGTAAAGAGGGCAGA	103	EF635410	
	CCRMxRRE	GAATCCAGGGCGAGTTTCC			

B-actin	CCRBACFW	TCACCACCACAGCCGAGAG	110	M24113	
	CCRBACRE	CAGGGAGGAGGAGGAAGCAG			
KIRV MCP	MCPFORW	TGATTGGCAACACTAGCG	156	KJ939444	George *et al.* [1]
	MCPREV	CGTTGTAAGGCAGGGTGA			

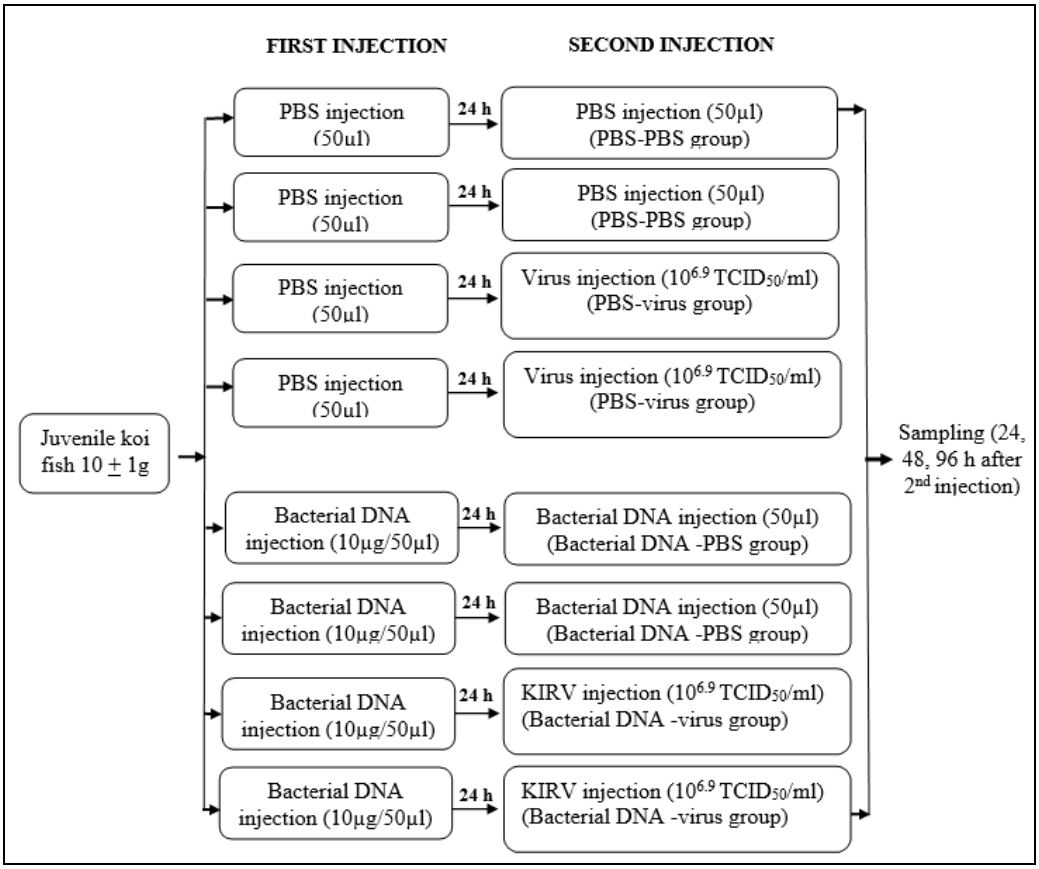

Fig 1: Experimental design. (Expression of immune genes in koi induced by bacterial DNA and koi ranavirus (KIRV).

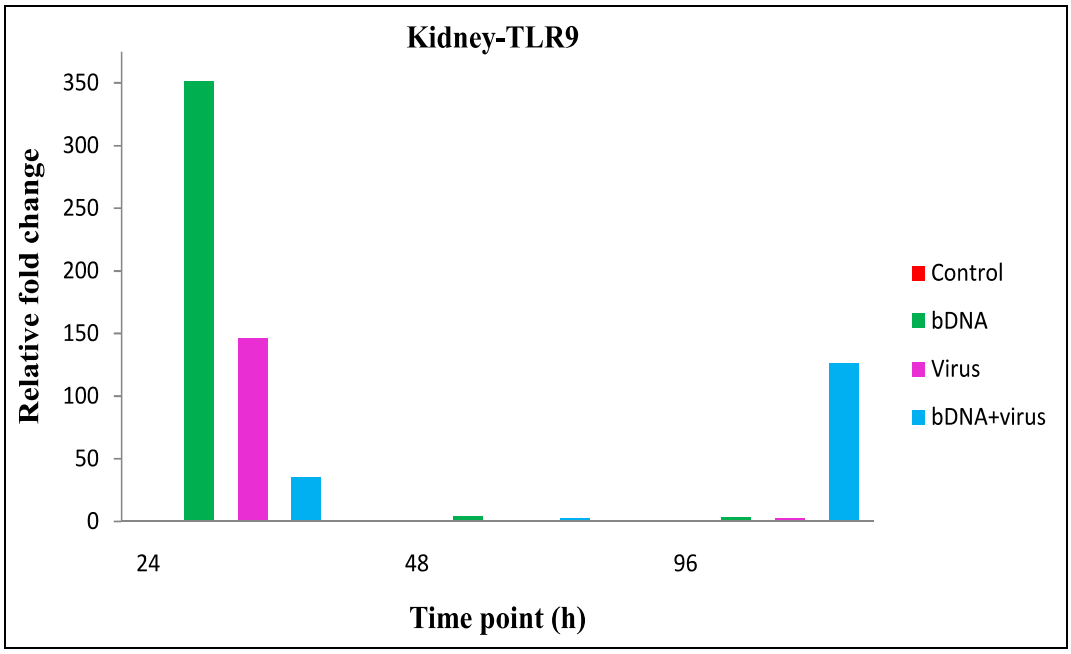

Fig 2: Effect of bacterial DNA pre-treatment on TLR9 mRNA levels (Relative fold induction ($2^{-\Delta\Delta CT}$) in kidney after koi ranavirus challenge. Samples were collected at 24, 48 and 96 h after the second injection.

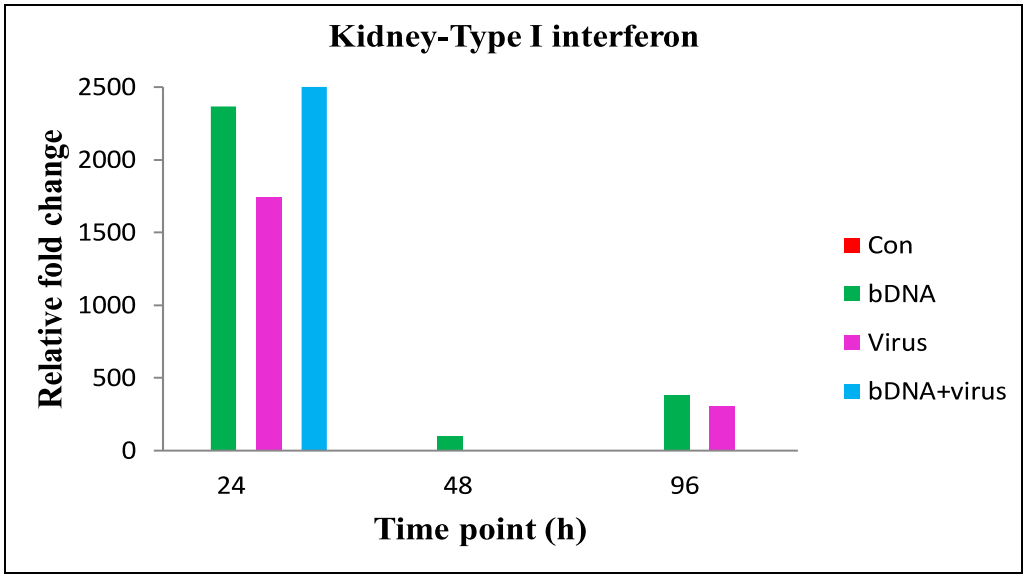

Fig 3: Effect of bacterial DNA pre-treatment on type I interferon mRNA levels (Relative fold induction ($2^{-\Delta\Delta CT}$) in kidney after koi ranavirus challenge. Samples were collected at 24, 48 and 96 h after the second injection.

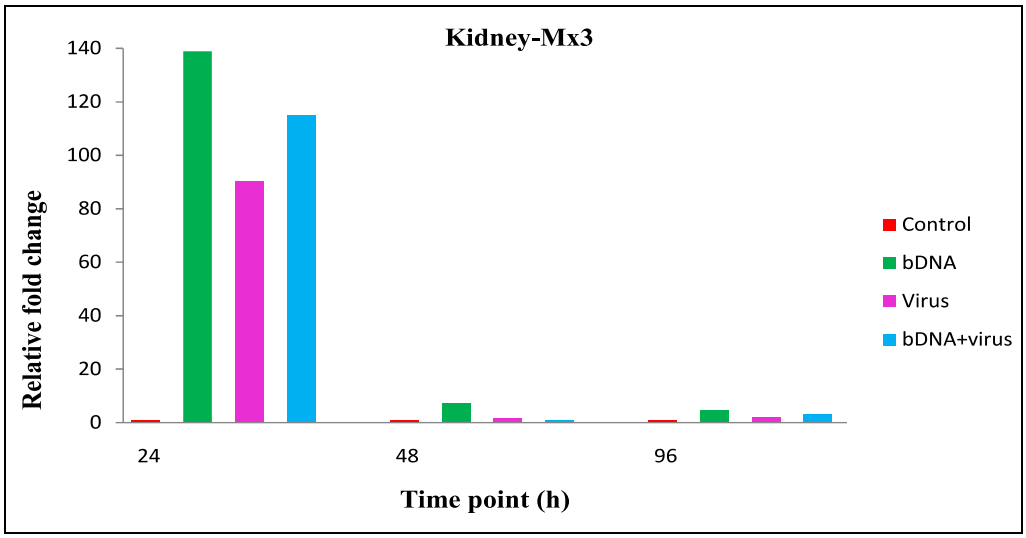

Fig 4: Effect of bacterial DNA pre-treatment on Mx3 mRNA levels (Relative fold induction ($2^{-\Delta\Delta CT}$) in kidney after koi ranavirus challenge. Samples were collected at 24, 48 and 96 h after the second injection.

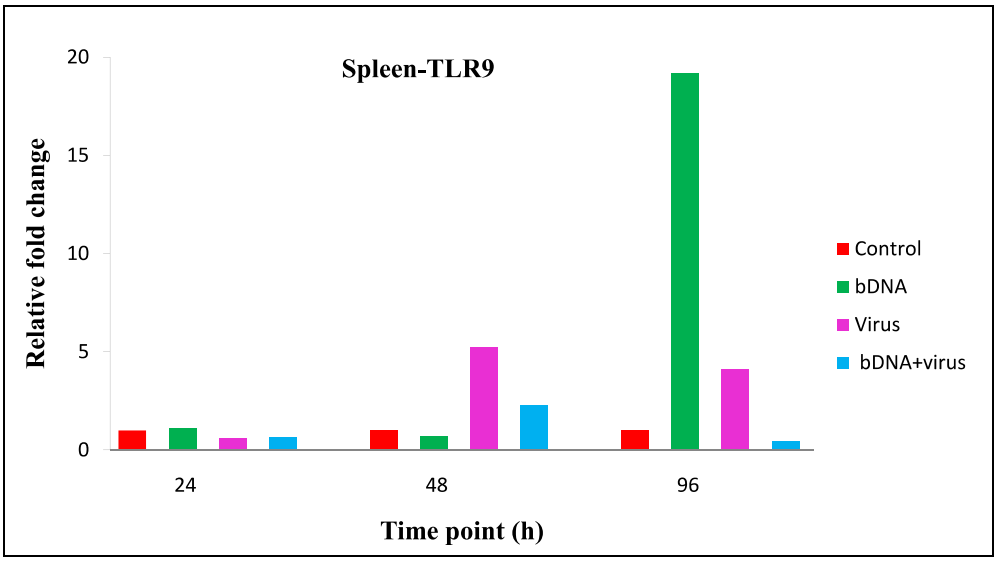

Fig 5: Effect of bacterial DNA pre-treatment on TLR9 mRNA levels (Relative fold induction ($2^{-\Delta\Delta CT}$) in spleen after koi ranavirus challenge. Samples were collected at 24, 48 and 96 h after the second injection.

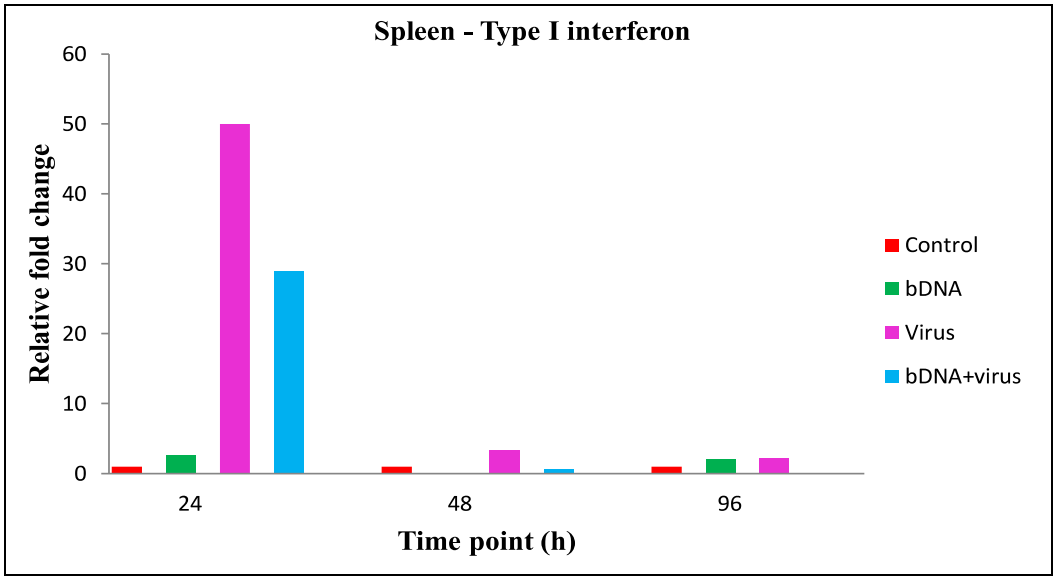

Fig 6: Effect of bacterial DNA pre-treatment on type I interferon mRNA levels (Relative fold induction ($2^{-\Delta\Delta CT}$) in spleen after koi ranavirus challenge. Samples were collected at 24, 48 and 96 h after the second injection.

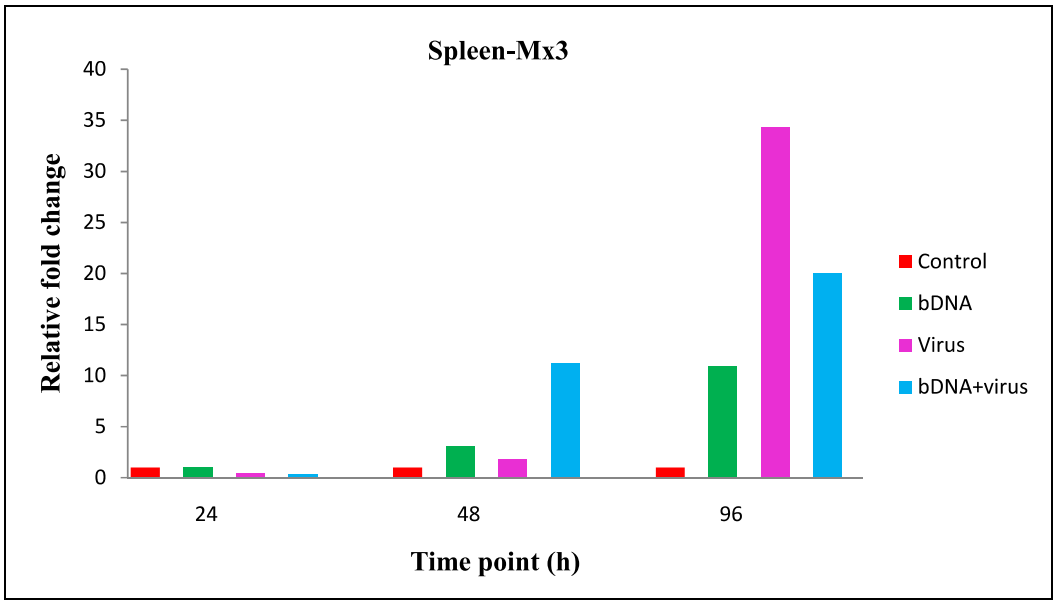

Fig 7: Effect of bacterial DNA pre-treatment on Mx3 mRNA levels (Relative fold induction ($2^{-\Delta\Delta CT}$) in spleen after koi ranavirus challenge. Samples were collected at 24, 48 and 96 h after the second injection.

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