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Administration of yeast glucan enhances survival and some non-specific and specific immune parameters in carp (*Cyprinus carpio*) infected with *Aeromonas hydrophila*

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Abstract

Effects of β-glucan administration on survival and immune modulations were studied in *Cyprinus carpio* against the bacterial pathogen, *Aeromonas hydrophila*. β-glucan was extracted from *Saccharomyces cervisiae* and purified. A virulent strain of the pathogen *A. hydrophila* was collected from infected fish. Different concentrations of β-glucan were administered to test animals on day 1, 3 and 5 through different routes (intraperitoneal injection (ip), bathing and oral administration). Control and test animals were challenged by ip injection of LD50 concentration of *A. hydrophila* on day 7 and mortality was observed and Relative Percent Survival (RPS) was calculated. Intraperitoneal injection of 500 μg of glucan significantly enhanced the RPS; bathing and oral administration of glucan did not influence the RPS. On day 7, test animals injected with 100, 500 and 1000 μg of glucan had a significant increase in total blood leucocyte counts and an increase in the proportion of neutrophils and monocytes. Superoxide anion production by kidney macrophages was also elevated. RT-PCR and northern blot analysis of interleukin-1 mRNA showed elevated expression in kidney on day 7 in fish injected with glucan. Glucan had an adjuvant effect on antibody production as pretreatment by injection of 100–1000 μg glucan/fish resulted in the highest antibody titer against *A. hydrophila* following vaccination. Classical and alternative complement pathways were not affected by glucan administration by any of the three routes.

Keywords: Carp; Macrophage; Phagocytosis; Opsonin; NBT assay; Complement; Antibody; IL-1β; *Aeromonas hydrophila*

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1. Introduction

Immunostimulants are widely used in farms for health management. Fish treated with immunostimulants usually show enhanced protection against various pathogens. This increased protection to infectious diseases is not by enhancing specific immune responses but by enhancing non-specific cellular and humoral defense mechanisms [1]. Yeast β-1,3 glucan has been shown to enhance the disease resistance against several major bacterial pathogens including *Vibrio anguillarum*, *Vibrio salmonicida*, *Yersinia ruckeri, Edwardsiella tarda* and *Aeromonas hydrophila* in several fish species such as carp *Cyprinus carpio* [2], Atlantic salmon *Salmo salar* [3], yellowtail *Seriola quinqueradiata* [4], rainbow trout *Oncorhynchus mykiss* [5], brook trout *Salvelinus fontinalis* [6], and African catfish *Clarius gariepinus* [7]. This increased resistance observed subsequent to the application of β-(1,3) D-glucan is mediated by enhancement of non-specific cellular as well as humoral defense mechanisms. Several workers have reported the effect of β-(1,3) D-glucan on non-specific cellular and humoral defense mechanisms including the function of phagocytes, bacterial killing activity of macrophages in rainbow trout, Atlantic salmon and catfish [5,8–15] and the production of superoxide anion by macrophages [14–16], and acceleration in the synthesis of cytokine-like molecules [17]. Interleukin 1 is one of the cytokines produced by macrophages in response to an immunogenic stimulus in fish. Previous studies revealed that glucan treatment in fish enhanced the expression of interleukin 1 [18] and complement activity [19] and also accelerated the specific immune response in catfish and Atlantic salmon against vaccines for *E. ictaluri* and *A. salmonicida* respectively [11,20].

For use in aquaculture, the mode of application of immunostimulants is very important and it should be acceptable with regard to labour input, vaccine consumption, and level of protection and stress of the fish. β-(1,3)-D-glucan is usually given by intraperitoneal injection; the drawback of injection is that it is labour intensive and also stressful to fish. Bathing and oral administration are potentially useful alternative methods for mass administration to fish of all sizes. However, there is very little information available on bathing and oral administration protocols or dosage in farmed fish species.

The aim of this work was to investigate the most effective route and dosage of yeast glucan by administering through injection, bathing and oral routes and its impact on relative percent survival (RPS) of carp, *C. carpio,* against the pathogen *A. hydrophila*. The RPS was correlated with hematological studies and functional assays performed under in vitro conditions. Bacterial killing, superoxide anion production, complement (classical and alternative pathway) activity, specific immune response against an *A. hydrophila* vaccine and level of expression of interleukin 1β mRNA were studied in the fish.

2. Materials and methods

2.1. Experimental animals

C. carpio were purchased from Manimutharu dam, Manimutharu, Tirunelveli District, Tamilnadu, and transported to the laboratory in aerated plastic bags. The animals were allowed to acclimatize to laboratory conditions for 15 days and then used for experiments. The weight of the animals ranged between 25 and 30 g. The experiments were carried out in glass tanks with 250 L capacity containing tap water at 30 ± 2 °C. The water was changed on alternate days. The fish were fed with a pelleted diet containing 35% crude protein, which was prepared in our laboratory.

2.2. A. hydrophila

The pathogen was isolated from infected *C. carpio*, collected from Manimutharu dam, Manimutharu, Tirunelveli District, Tamilnadu, according to Shome and Shome [21]. Based on the comparative

biochemical tests, the isolated bacterium was identified as *Aeromonas hydrophila*. To determine the LD50 concentration of the pathogen, the bacterium was cultured in the laboratory in LB broth at 37 °C for 24 h then the cells were separated by centrifugation at 8000×g and ip injected in carp (size 25 to 30 g) at room temperature by following the method of Saeed and Plumb [22].

2.3. β-Glugan preparation

β-glucan was isolated from yeast *Saccharomyces cerevisiae* and characterized following published methods [23,24]. Purified β-glucan was suspended in phosphate buffered saline (PBS, pH 7.2) and subjected to sonication to reduce its particle size.

2.4. Administration of β-glucan

2.4.1. Intraperitoneal route

Fish were taken from the stock tank and divided into four groups. Each group was comprised of 24 fish. Three groups received the β-glucan by intraperitoneal (ip) injection at three different concentrations (100, 500 and 1000 μg/fish) on day 1, 3 and 5. The fourth group (control) received 0.1 ml PBS only.

2.4.2. Bathing route

β-glucan containing baths were prepared with different concentration of 150, 750 and 1500 μg ml^{-1}. Each group comprised 24 fish. Test fish were immersed in the aerated glucan solution for about 90 min on days 1, 3 and 5. Control fish were immersed in tap water on day 1, 3 and 5.

2.4.3. Oral route

Experimental diets were prepared for oral administration by mixing the glucan with lab made basal diet. Pelleted feed containing 1, 2 and 4% of glucan were fed to three groups of the test animals twice a day on days 1, 3 and 5. Control animals were fed lab-made basal diet without the glucan. Each group comprised 24 fish.

2.4.4. Challenge study

All test and control groups comprised 24 fish, they were challenged by i.p injection of LD50 concentration of *A. hydrophila* on day 7 and mortality was recorded daily up to 7 days and Relative percent survival (RPS) was calculated following the method of Baulny et al. [20].

$$\mathrm{RPS}(\%) = \frac{\text{Mortality (\%) of untreated controls} - \text{Mortality (\%) of treated}}{\text{Mortality (\%)of untreated controls}} \times 100$$

2.5. In vitro assays

Blood samples were taken from 6 animals of each group of glucan treated and control group for hematology, antibody determination and complement assay. Kidney was removed to study bacterial killing, NBT and interleukin 1β mRNA assay on day 7.

2.6. Hematology

2.6.1. Total leucocyte count (TLC)

Total Leucocytes Counts were made from 6 animals of each group in a Neubauer counting chamber as described by Ref. [25].

2.6.2. Differential count

Blood smears were stained from 6 animals of each group with *May-Grunwald/Giemsa* and 100 leucocytes were counted under the microscope and the percentage of different types of leucocytes was calculated following the method of [25].

2.6.3. Isolation of anterior kidney macrophages

Macrophages were isolated from kidney as described by [26]. The kidney of the fish was dissected out and transferred to 7 ml of L-15 medium supplemented with 10% foetal calf serum (FCS). A cell suspension was prepared by pressing the kidney with a glass rod through a stainless steel mesh (diameter 0.3 mm) in a plastic petri dish on ice.

Cells were suspended in L-15 (HI Media, India) supplemented with 0.33 g ml^{-1} glucose, 100 IU ml^{-1} penicillin-streptomycin and 10% FCS. The medium was adjusted to pH 7.6 and sterilized by syringe filtration and 10 IU ml^{-1} heparin (sterile) was added to the medium.

Cell suspensions were loaded onto a discontinuous (densities 1.08 and 1.07) Percoll gradient (Sigma) and centrifuged for 40 min at 400×g at 4 °C. The macrophage enriched fraction was collected and the cell number counted in a hemocytometer. The cells were washed in L-15 medium twice by centrifugation, the supernatant discarded and the pellet resuspended in L-15 medium containing 10% FCS at a concentration of 1×10^6 cells ml^{-1}.

2.6.4. Bacterial killing assay

This was performed according to Chen and Ainsworth [11]. A bacterial concentration of *A. hydrophila* of approximately 1×10^7 cfu ml^{-1} was used as a stock. From this, 0.1 ml was taken and mixed with 0.1 ml of macrophage suspension (1×10^6 cell ml^{-1}) and 0.04 ml of pooled fresh carp serum collected from 10 animals was added, mixed well and incubated for 2 h with occasional shaking in a water bath at 27 °C. After 2 h, 0.1 ml of the bacteria-macrophage mixture was diluted with 9.9 ml of sterile distilled water to release living bacteria from phagocytes. This was serially diluted, plated on LB agar plates, incubated overnight at 37 °C and the number of colonies was counted.

2.6.5. Zymosan A activation

This experiment was performed by the method of Ref. [12]. Zymosan A (Sigma) was suspended in 0.9% NaCl (10 mg ml^{-1}), boiled in a water bath for 30 min, washed twice in PBS and opsonized by incubation with fresh carp serum (5 mg ml^{-1}) for about 1 h at 14 °C. The coated zymosan was then washed twice in PBS and diluted to 10 mg ml^{-1} in PBS and stored in small aliquots at −20 °C.

2.6.6. Oxygen burst activation assay

This experiment was performed as described by Refs. [27,28]. From the macrophage suspension ($\sim1\times10^6$ cells ml^{-1} in L-15 medium), 100 μl was placed in a 96 well microtiter polystyrene plate and allowed to adhere for 2 h at 18 °C. Non-adhered cells were removed by three washes with 100 μl/well of L-15 culture medium supplemented with 10% FCS. To the macrophage monolayer, 100 μl/well of NBT solution (1 mg ml^{-1} L-15, 10% FCS) containing activated opsonized Zymosan A at 500 μg ml^{-1} was added. After 30 min incubation at 12 °C, the medium was removed and the culture was washed twice with isotonic PBS, fixed with 100 μl/well of 100% methanol for 3 min, washed twice with 70% methanol and the cells

were air dried. Formazan was solubilized in 120 μl of KOH (2M) and 120 μl of DMSO (100%) and the absorbance was read spectrophotometrically (Hitachi) at 620 nm using KOH/DMSO as blank.

2.6.7. RNA preparation

Macrophages separated by gradient centrifugation were washed twice with PBS at 400 g at 4 °C for 5 min; the pellet lysed with Trireagent (Sigma) and the RNA prepared according to the manufacturer's protocol. Finally, the RNA pellet was dissolved in 30 μl of DEPC-treated water. The concentration of the total RNA was assayed spectrophotometrically at 260 nm (Hitachi).

2.6.8. Preparation of cDNA

RNA (5 μg), 1 μl of oligo dT primer (Promega, USA), 1 μl of 10 mM dNTP mix and 10 μl of DEPC water were mixed and incubated at 65 °C for 5 min, placed on ice for 1 min and then the pellet was collected by centrifugation at 8000×*g*. To this pellet, the following components were then added: 4 μl of 5×first strand buffer, 1 μl of 0.1M DTT and 1 μl of RNAsin (40u μl ml^{-1}) (Promega, USA) and mixed gently and incubated at 42 °C for 1 min and 1 μl of (200u) of MMLV reverse transcriptase enzyme (Gibco BRL, USA) was added and incubated at 42 °C for 50 min. The reaction was terminated by keeping the content at 70 °C for 15 min and by placing the sample on ice for 1 min and then kept at −20 °C until further use.

2.6.9. PCR assay

The synthesized first strand cDNA (2 μg) was taken from each sample. PCR reaction was conducted in 20 μl, which contained 1 μl of the first strand cDNA, 0.8 μl of 5 μM of specific forward and reverse primer, 0.4 μl of 10 mM dNTPS mixture, 0.5 μl of Taq polymerase, 0.8 μl of 2 mM $MgCl_2$ and 2 μl of 1×Buffer and 13.7 μl of sterile mille Q H_2O. The PCR reaction was performed in a thermocycler (Mini cycler, MJ Research, USA) under the following conditions: 95 °C, 5 min: 40 cycles at (95 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min) and a final extension at 72 °C for 5 min. Oligonuleotides used as PCR primers were as follows: carp 40 s ribosomal protein S11 (433 bp) sense primer 5′TACAGAACGAGAGGGCTTATC (13–33) antisense primer 5′TTGGTGACCTTCAGGACATTG (425–445); carp IL-1β (574 bp) sense primer 5′-ACCACTGGATTTGTCAGAAG (76–96), antisense primer 5′-ACAGGGGAAGAACCTGTCATTT CAG (629–649). An aliquot (10 μl) of the PCR product was electrophoresed on a 1.5% agarose gel containing 0.5 μg ml^{-1} of ethidium bromide photographed on a UV illuminator and intensity of each band was quantified by alpha image documentation and analysis system (Alpha Innotech Corporation, USA).

2.6.10. Northern hybridization

Total RNA was extracted and purified. RNA samples were prepared for northern hybridization as described by Sambrook et al. [29] by mixing RNA sample with sample buffer, which contained deionized formamide, 37% formaldehyde and 5×MOPS. Samples were then heated at 65 °C for 5 min, cooled to room temperature and RNA loading buffer (50% glycerol, 1mM EDTA and 0.4% bromophenol blue) was added. The RNA samples were fractionated on 1% agarose gel. The gel running buffer was 1×MOPS prepared in sterile DEPC water.

After electrophoresis, the gel was soaked in DEPC water to remove the formaldehyde and then washed in 20×SSC for 45 min. The RNA in the gel was blotted to a nylon membrane by capillary action. The membrane was removed and washed once in 5×SSC for 5 min at room temperature and the RNA was immobilized on to the membrane by UV cross-linking. The membrane was wetted in 5×SSC and placed in a heat sealable container and subjected to prehybridization at 42 °C for 2 h. The DNA probe was prepared from RT-PCR amplified specific DNA. The DNA was extracted from an agarose gel by DNA extraction kit (Qiagen). For radio-labeling 1 μl 50 ng ml^{-1} DNA was placed in a clean autoclaved eppendorf tube and 9 μl of milli Q water was added and boiled for 5 min to denature. Then it was immediately chilled on ice for 5–10 min and centrifuged at 5000×*g* for 30 sec. The following reagents were then added: 2.5 μl of

10× labeling buffer, 1 μl of 100-ng μl^{-1} of random primer, 2.5 μl of 20 mM DTT solution, 2.5 μl of 20 mM dNTP solution, 3 μl of $\alpha[P^{32}]$ dCTP (Sp. activity >3000 Ci/mMole or 10 μCi/μl), and 3 μl of nuclease free autoclaved water. To this, 1 μl of 3u μl^{-1} of klenow (large fragment of DNA polymerase I) was added and mixed gently. The mix was incubated at room temperature for at least 2 h. After labeling, the probe was briefly boiled for 5 min and then cooled immediately in ice for 10 min. Then the blot was hybridized at 42 °C overnight in a solution containing 1% SDS, 1 M NaCl and 3×10^5 cpm ml^{-1} of DNA probe. After hybridization, the blot was washed twice with 2×SSC containing 0.1% SDS followed by three washings at 68 °C and finally washed with 2×SSC for 30 min at room temperature. The blot was wrapped and autoradiography was performed.

2.6.11. Preparation and purification of immunoglobulin from carp anti-Aeromonas hydrophila serum

The vaccine was prepared from *A. hydrophila* at a concentration of 2.4×10^7 CFU ml^{-1} and heat inactivated at 65 °C for about 1 h and emulsified with an equal volume of Freund's incomplete adjuvant (Sigma USA). Fish were ip injected with 0.1 ml of vaccine on day 1 and boosted on day 7. Five days after booster injection, blood samples were collected from caudal vessels, allowed to clot and serum collected. Immunoglobulin (Ig) was purified from this antiserum by following the method of Waterstrat et al. [30]. The anti-serum from several fish was pooled and precipitated with 50% ammonium sulphate solution. The precipitate was dissolved in PBS and desalted by dialyzing overnight against PBS and concentrated on an Amicon ultra filtration apparatus (Amicon, USA). The protein concentration in the sample was determined by Bradford's method [31]. The concentrated protein was placed on a 1.6×92 cm Sepharose 6B column (Pharmacia) and eluted with PBS at a flow rate of 0.33 ml/min and 100 fractions were collected. The O.D was taken for each fraction at 280 nm. The peak OD fractions were collected and tested for agglutination against *A. hydrophila* at a concentration of 2.4×10^7 CFU ml^{-1}. The fraction with peak agglutinating activity was tested for purity by SDS-PAGE by following method of Laemmli [32].

2.6.12. Rabbit anti-carp Ig production

Rabbit anti-carp immunoglobulin was prepared according to the procedure of Murai et al. [33]. Rabbits weighing about 2 Kg were given two subcutaneous injections at two-week interval of 500 μl of purified carp Ig (1 mg ml^{-1} PBS) emulsified in an equal volume of Freund's Complete Adjuvant (FCA). A third injection of carp Ig without FCA was given 4 days later and serum was collected after four days.

The rabbit antiserum was titrated for anti-carp Ig activity by ELISA. Briefly, purified carp Ig was diluted in carbonate buffer at concentrations of 2.5 and 5 μg protein ml^{-1} and 50 μl/well was placed in a 96 well microtiter-plate and incubated overnight at room temperature (25 °C). The plates were washed three times in PBS containing 0.05% Tween 20 (PBST). To duplicate wells, 100 μl of rabbit anti-carp Ig (RACI), previously doubly diluted from 1:1 to 1:16,384 with 1% milk powder in PBS was added and then washed twice with PBS. Fifty μl of goat anti-rabbit IgG HRP conjugate (Bangalore Geni), diluted to 1:2000 in PBS, was added to each well, incubated for 60 min and then wells were washed three times in PBS. One hundred μl of substrate Tetramethyl benzodine TMB/H_2O_2 diluted in PBS was added to each well and incubated for 45 min in a dark room and 50 μl of 0.1N H_2SO_4 was added as a stopping reagent. The color reaction was evaluated at 450 nm with an ELISA reader (Biorad). Result from the check board titration of rabbit anti-carp Ig antiserum indicated that the optimal dilution was 1:2048 at a coating concentration of 5 μg ml^{-1} protein.

2.6.13. Adjuvant effect of glucan on antibody response to A. hydrophila

Glucan treated and control fish were injected ip with *A. hydrophila* vaccine in Freund's incomplete adjuvant (FIA) (Sigma) on day 7 and the booster without FIA was given on day 14. On day 21 blood was drawn; the serum was separated and assayed for antibody titer by ELISA following the method of

Waterstrat et al. [30]. Briefly, 50 μl of heat killed *A. hydrophila* (7×10^7 CFU mL^{-1}) in PBS, were coated onto flat bottomed 96 well polystyrene microtiter plates which were previously coated with 50 μl of a poly-L-lysine solution (1 mg/100 ml in PBS) (Sigma), for 2 h at room temperature. The bacteria were fixed with 50 μl of cold 0.5% glutaraldehyde solution. After 15 min incubation, the plates were washed twice in PBST. Excess glutaraldehyde was blocked by addition of 100 μl of a solution containing 100 mM glycine and 1% bovine serum albumin in PBS and incubated for 30 min. Following incubation, the plates were washed three times in PBST and dried under a heat lamp.

Carp serum was pooled from 6 animals of each group and serially diluted from 1:1 to 1:16384 in 1% milk solution in PBS and incubated for 15 min, and 50 μl of appropriately diluted carp serum was added to duplicate bacteria coated wells and incubated for 60 min. Plates were then washed twice in PBST and 50 μl of RACI diluted 1:2048 in PBS 1% milk was added, incubated for 60 min and then washed twice with PBS. Fifty μl of goat anti-rabbit IgG HRP conjugate (Bangalore Geni), diluted to 1:2000 in PBS, was added to each well, incubated for 60 min and then wells were washed three times in PBST. One hundred μl of substrate Tetramethyl benzodine TMB/H_2O_2 diluted in PBS was added to each well and incubated for 45 min in a dark room and 50 μl of 0.1N H_2SO_4 was added as a stopping reagent. The color reaction was evaluated at 450 nm with an ELISA reader (Biorad).

2.7. Alternative complement pathway (ACP) activity

2.7.1. Preparation of sheep red blood cells (SRBC)

Sheep blood was mixed with equal volume of Alsever's solution and stored at 4 °C. Subsequently, cells were centrifuged at $400\times g$ for 5 min; the pellet of SRBC was washed twice in 10 mM EGTA-Mg-gelatin veronol buffer (GVB) and suspended in the same buffer at a concentration of 2×10^6 cells ml^{-1} for alternative complement pathway assay.

2.7.2. Serum collection

Blood was collected from the caudal vessels of 6 animals of each group and kept at 30 °C for 60 min and subsequently left in a refrigerator for 2 h. The fresh serum was separated by centrifugation at 400×g for 10 min and serum samples pooled.

2.7.3. Assay of alternative complement pathway (ACP)

ACP activity was assayed according to published methods [34,35]. Briefly, 0.5 ml of serially diluted carp serum in EGTA-Mg-GVB was placed in a set of test tubes and 0.2 mL of SRBC suspension (2×10^6 cells ml^{-1}) was added. This mixture was incubated at 15 °C for 90 min. Addition of 2.8 ml of 10 mM EDTA-GVB buffer stopped the hemolytic reaction. After centrifugation, the value y (percent hemolysis/100) was calculated from the optical density at 414 nm of the supernatant. The value $y/(1-y)$ and the reciprocal of the serum dilution were plotted on log–log graph paper and the ACH50 (units ml^{-1}), the reciprocal dilution giving 50% hemolysis ($y\ (1-y)=1$), was read from the graph.

2.8. Assay of classical complement pathway (CCP)

2.8.1. Carp anti-SRBC serum

C. carpio (250–300 g) were intraperitoneally injected with 0.25 ml of SRBC at a concentration of 2×10^8 cells ml^{-1} in PBS. One week later, a booster injection was given. After a further five days, blood was collected and the serum was separated and carp anti-SRBC antibody was determined by haemagglutination and incubated at 50 °C for 15 min to inactivate the complement as described by Sakai [36]. SRBC were washed three times with a GVB^{2+} Gelatin veronal buffer saline containing Mg^{2+} and Ca^{2+} ions and

resuspended to 2×10^8 cells ml^{-1} in GVB^{2+} buffer. SRBC were incubated with heat-inactivated carp anti-SRBC serum diluted at a ratio of 1:50, and incubated at 30 °C for 30 min with shaking every 5 min.

The test samples of serum were first diluted 1/50 with GVB^{2+} buffer and the diluted serum (0.3, 0.4… 0.8 ml) were mixed with GVB^{2+} Gelatin veronal buffer saline containing Mg^{2+} and Ca^{2+} ions (1 ml, 0.9 ml… 0.5 ml) and 0.2 ml of sensitized SRBC suspension was added to each tube. Complete hemolysis was obtained by mixing 1.3 ml of distilled water with 0.2 ml of sensitized SRBS (positive control). The negative control contained 0.2 ml of sensitized SRBC mixed with 1.3 ml of GVB^{2+} Gelatin veronal buffer saline containing Mg^{2+} and Ca^{2+} ions after which it was incubated at 20 °C for 60 min and then centrifuged at $800\times g$ for 5 min. The optical density of the supernatant was measured at 541 nm. A lysis curve was obtained by plotting the percentage of hemolysis against the volume of serum added (ml); the volume which yielded 50% hemolysis was determined and used for calculating the complement activity of the samples (CH50 unit ml^{-1}) as described by Sakai [36].

3. Results

3.1. Relative percent survival (RPS) of carp challenged with A. hydrophila

The mortality rate was 46%, 54% and 50% for ip, bathing and oral control groups respectively. The RPS was significantly increased in fish injected with 500 μg and 1 mg glucan/fish (Table 1). Bathing and oral administration of glucan did not induce any change in the survival rate compared with their control groups.

3.2. Total leucocyte count (TLC) and differential count

Glucan injected fish ($n=6$) showed significantly increased TLC, which was directly proportionate to the dose of glucan injected. Among the leucocytes, neutrophils increased significantly in glucan injected groups and were predominant, followed by monocytes. Other leucocytes like eosinophils and basophils declined in number. The lymphocyte count did not vary much between the control and experimental groups (Table 1). Total leucocyte count and differential count did not change in the bath and oral administrated groups.

Table 1
Effect of glucan administration by injection on relative percent survival (RPS) against LD50 concentration of *A. hydrophila* and other parameters of carp

	Concentration of glucan (μg/animal)			
Functional assay	PBS (control)	100	500	1000
Relative percent survival (RPS)	–	71.4	100	100
NBT assay OD 620 nm 1×10^6 cell/ml	0.20 ± 0.015	$0.30\pm0.005^{*}$	$0.38\pm0.005^{*}$	$0.37\pm0.015^{*}$
Bacterial killing assay CFU/ml	$268\times10^3\pm10.066$	$65\times10^2\pm7.435^{**}$	$48\times10^2\pm3.511^{**}$	$17\times10^2\pm4.163^{**}$
TLC (n. $\times10^3/mm^3$)	24 ± 0.816	$35\pm0.816^{**}$	$44.66\pm1.247^{**}$	$48.44\pm0.471^{**}$
Neutrophil %	24.0 ± 0.816	$35\pm0.18^{**}$	$43.0\pm0.471^{**}$	$46.3\pm0.471^{**}$
Monocytes %	22.66 ± 0.471	30.33 ± 0.471	$31.0\pm0.816^{*}$	$30.6\pm0.816^{*}$
Lymphocyte %	23.66 ± 0.942	26.0 ± 0.816	23.33 ± 0.471	23.0 ± 0.816
Basophil %	18.0 ± 0.816	3.0 ± 0.816	0	0
Eosinophil %	12.66 ± 0.471	5.66 ± 0.942	1.0 ± 0.816	0

* Significant at 5% level, ** significant at 1% level.

3.3. Bacterial killing assay

A. hydrophila was killed more efficiently by macrophages of fish given intraperitoneal injection of glucan than macrophages of fish treated with glucan by bathing or oral administration. Bacterial counts were significantly reduced ($t=26.6$, $n=6$; $P<0.01$) even at the lowest glucan concentration (100 μg/ fish) given by intraperitoneal injection (Table 1), while other routes of administration did not show significant change (bathing; $t=1.799$, $n=6$; $P>0.05$; oral: $t=1.282$, $n=6$; $P>0.05$) compared with the control.

3.4. Superoxide anion production/NBT assay

Fish treated with glucan by intraperitoneal injection at all doses, showed significant increase ($t=2.168$, $n=6$; $P<0.05$) in superoxide anion production compared to the control (Table 1). Administration of glucan by the other two methods did not induce any significant change (bathing: $t=0.150$, $n=6$; $P>0.05$; oral route: $t=1.315$, $n=6$; $P>0.05$) when compared with control animals.

3.5. RT-PCR analysis assay

RT-PCR analysis assay revealed that the expression of IL-1β mRNA in macrophages of control fish was at a steady state in both samples taken at 24 and 48 h, whereas the fish injected with glucan at all doses showed enhanced expression of IL-1β mRNA over the control (Fig. 1). 40S ribosomal protein was used as a positive control to confirm the total RNA concentration, which indicated that this was almost the same in all samples. In glucan-injected fish, the band densities were higher than in control fish at both 24 h and 48 h.

3.6. Northern blot analysis

In this experiment, IL-1β mRNA was confirmed by using a carp RT-PCR amplified specific radioactively labeled DNA probe. Analysis revealed that IL-1β mRNA signal was detected in fish injected with glucan (Fig. 2).

3.7. Antibody purification

Carp anti- *A. hydrophila* immunoglobulin was separated and purified by chromatography and this was confirmed by SDS-PAGE. Bands appeared at approximately 73 kDa as heavy chain and 23 kDa as light chain. No other band appeared in the electrophoretogram (Fig. 3).

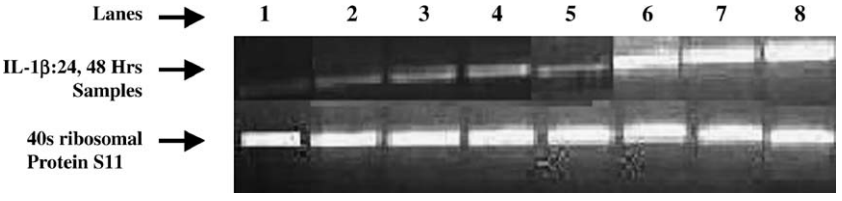

Fig. 1. RT-PCR analyzed mRNA expression of Carp IL-1β in kidney macrophages of fish injected with glucan. Samples were taken for analysis after 24 h and 48 h of the final glucan injection. Lane 1=PBS; Lanes 2, 3 and 4, 24 h samples of 100, 500 and 1000 μg glucan/ fish respectively; Lane 5=PBS; Lanes 6, 7 and 8, 48 h samples of 100, 500 and 1000 μg glucan /fish.

Fig. 2. The expression of Carp IL-1β mRNA following injection of different doses of glucan. The expression was confirmed by Northern blot analysis probed with P^{32} labeled with RT-PCR amplified specific DNA probe. Lane 1–100 μg of glucan, Lane 2–500 μg of glucan and Lane 3–1000 μg of glucan administration.

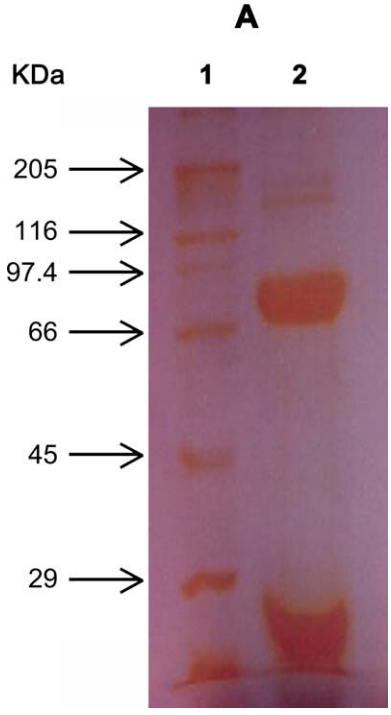

Fig. 3. SDS-PAGE analysis of the purified carp-Ig from carp serum. Sample run on a 10% SDS-PAGE gel. Lane 1 – molecular weight marker. Lane 2 – sample.

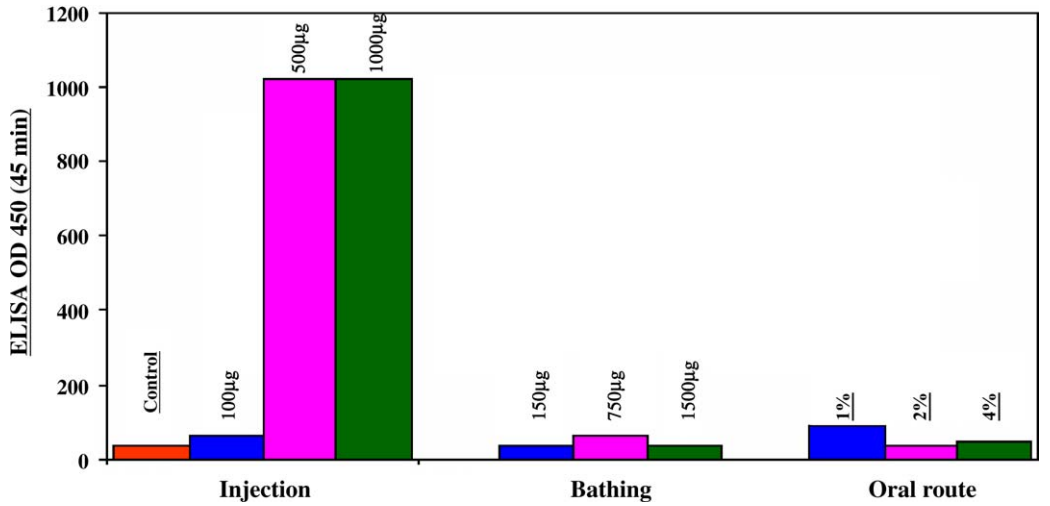

Fig. 4. Antibody titer against *A. hydrophila* of *C. carpio* pretreated with different doses of glucan by injection (100, 500 and 1000 µg/fish), bathing (150, 750 and 1500 µg/ml) and oral route (1, 2 and 4%) following vaccination with *A. hydrophila*. Serum samples pooled from 6 fish.

3.8. Adjuvant effect of glucan

Fish ip injected with glucan showed higher antibody titer following vaccination with *A. hydrophila* than the untreated control. The antibody titer of pooled ($n=6$) control and test animals injected with 0, 100, 500 and 1000 µg glucan were 1:32, 1:64, 1:1024, and 1:1024 respectively. The antibody titer was almost similar in test and control fish when glucan was administered by the bathing method. The antibody titer was slightly increased when 1% glucan was orally administered compared with immersion method (Fig. 4).

3.9. Complement activity assay

3.9.1. Classical pathway

The mean CH 50 unit ml^{-1} of the control group was 40.8 ± 1.3. There was no significant ($P > 0.05$) difference in CH50 unit ml^{-1} between the serum of glucan treated and control animals.

3.9.2. Alternative complement pathway

Glucan treatment through injection, bathing and oral route did not induce any change ($P > 0.05$) in this activity, which for the control group was mean 560 ± 32.2 ACH 50 unit ml^{-1}.

4. Discussion

The present study revealed that intraperitoneal injection of yeast glucan significantly improved the survival rate of *C. carpio* against an ip challenge with the pathogen *A. hydrophila*. Administration of 500 and 1000 µg glucan/fish elicited 100% survival in *C. carpio*. Previous workers have observed increase in survival rate of fish treated with glucan. Robertson et al. [3] reported that intraperitoneal injection of yeast

glucan (β-1,3 and β-1,6 linked glucan) prepared from the cell wall of *Saccharomyces cerevisiae*, resulted in increased resistance in Atlantic salmon ip challenged with *V. anguillanum, V. salmonicida and Y. ruckeri*. Catfish injected with yeast glucan showed increased resistance against ip challenge with *E. ictaluri* [11]. Our study revealed that glucan administration through bathing and oral routes, however, did not improve the survival percentage of *C. carpio*. Chinook salmon immersed in glucan solution did not show protection against bath challenge with *Aeromonas salmonicida* [10].

The protective effect of glucan injection was correlated with several functional assays under in vitro conditions. The total blood leucocyte number was increased in glucan-injected fish. The highest leucocyte number was found in fish injected with 1000 μg glucan/fish compared to the control group. Among the leucocytes, neutrophils and monocytes had increased significantly. The present results concur with the reports of previous workers [5,6,16].

The ability of macrophages to kill pathogenic microbes is probably one of the most important mechanisms of protection. The present study was performed with macrophages obtained from fish treated with glucan by different modes of administration. Intraperitoneal injection of glucan enhanced superoxide anion production that may be involved in the destruction of *A. hydrophila*. This was correlated with the bactericidal assay where the survival of *A. hydrophila* exposed to macrophages from fish pretreated with 1000 μg of glucan/fish was dramatically reduced compared to exposure to macrophages from control fish. However, animals receiving glucan by bathing or oral routes did not show any change in the above parameters. Many previous workers have also reported enhanced bacterial killing activity in glucan treated rainbow trout and Atlantic salmon [7,12].

RT-PCR and Northern blot analyses were done to determine the pattern of expression of IL-1β in response to glucan administration by injection. Fujiki et al. [18] reported that IL-1β expression is induced by stimulation of carp head kidney macrophages with the application of sodium alginate and scleroglucan. The present results indicate that interleukin 1β mRNA gene transcription in carp was also induced by yeast glucan. Expression of interleukin 1β mRNA was higher in glucan treated fish and the level of IL-1β mRNA depended upon the dose of glucan, the highest expression being found in fish sampled at 48 h following the last injection of 1000 μg of glucan/fish.

The present investigation showed that injection of the higher concentrations of yeast glucan prior to vaccination evoked higher antibody titers following vaccination. The minimum amount of glucan required for enhanced production of antibody was 500 μg/fish (Fig. 4). Chen and Ainsworth [11] and Aakre et al. [37] reported that intraperitoneal injection of yeast glucan in catfish and salmon increased the antibody response to vaccination with *E. ictaluri* and *A. salmonicida*. In the present study, animals receiving glucan by bathing and oral routes did not show any enhancement in the antibody response. Similar observations have been reported by others [10,38].

Complement, another component of the non-specific humoral immune response, was also studied in the present work. β-glucan did not induce any change in the classical or the alternative pathways when compared with control animals. Other studies also found that oral administration of β-glucan to turbot did not induce any change in classical complement pathway [20].

Among the three routes of administration, the advantage in injection method is that glucan can reach the target organs and induce the macrophages to enhance the non-specific cellular immune response. Fish probably cannot absorb glucan particles through the bathing route, as they are insoluble in nature. Although glucan particles are supposed to be absorbed through the intestinal wall when administered orally, only particles less than one micron might be capable of passing through the walls of the intestine and blood capillaries.

It may be concluded from the results obtained from this work that intraperitoneal injection of glucan in carp, enhanced resistance to an injection challenge by *A. hydrophila* and many aspects of non-specific and specific immune responses. However, bathing and oral administration of glucan did not induce any change in the parameters.

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