Innate immune responses in families of Indian major carp, *Labeo rohita*, differing in their resistance to *Edwardsiella tarda* infection

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Genetic variation in the innate immune responses of Indian major carp, rohu (*Labeo rohita*) families was investigated. Innate immune responses were measured among the fullsib families of rohu, produced using nested mating design for increased growth. Significant differences as well as large variations in each of the immune parameters were observed. The natural resistance of each family to *Edwardsiella tarda* infection was also assessed by intraperitoneal challenge study. A wide variation in per cent survival (0 to 100) was noticed among the families after bacterial challenge. A positive significant ($P < 0.05$) correlation was observed between per cent survival and superoxide production as measured through nitroblue tetrazolium assay. On the other hand, no significant correlation was observed between other immune parameters, viz. serum lysozyme activity, serum myeloperoxidase content, plasma glucose content, serum haemolysin, haemagglutination and bacterial agglutination titres measured and the per cent survival.

Keywords: Disease resistance, *Edwardsiella tarda*, innate immune responses, rohu.

EDWARDSIELLOSIS in Indian major carps poses one of the major threats to fish culture[1]. It is characterized by a generalized septicaemia. The symptoms include small cutaneous lesions which develop into necrotic abscesses on the ventral body surface, distended abdomen and swollen anus[2]. Chemotherapeutic treatment of this disease is of limited use due to the increasing resistance of the pathogen[3]. Development of a commercial vaccine for this disease is not yet available.

Under such conditions, the production of edwardsiellosis-resistant strains of fish, which is possible through selective breeding seems to be most promising. Selective breeding depends on the level of genetic variation for disease resistance to a pathogen within the population. A wide genetic variation in resistance has been noted for furunculosis in Atlantic salmon[4–8], *Edwardsiella ictaluri* infection in channel catfish[9], viral haemorrhagic septicaemia in rainbow trout[10], aeromoniasis in Nile tilapia[11], *Aeromonas hydrophila* infection in *Labeo rohita* (Ham.)[12] and *Gyrodactylus salaris* infection in Atlantic salmon and rainbow trout[13]. Direct selection of a strain based on challenge test and performance of the family has been described by many workers[6,8,14]. Indirect selection based on measurable traits on an individual basis that are genetically correlated to disease resistance was described by Roed *et al.*[15]. A strong correlation between non-specific immune parameters, viz. serum lysozyme, superoxide production, serum bactericidal activity, haemolytic activity, total level of IgM, phagocytic activity as well as specific immune parameter such as antibody titre, and disease resistance has been noted by earlier workers[7,11,12,16,17]. Camp *et al.*[9] noted that the resistant family of channel catfish against *E. ictaluri* produced more macrophage aggregations in spleen and posterior kidney than the sensitive family. A significant ($P < 0.05$) positive correlation was observed by Sahoo *et al.*[12] in *L. rohita* between serum bactericidal activity and survival against aeromoniasis. In mammals, natural antibodies (Nabs) are mostly of the IgM isotype and have the ability to bind to a particular antigen or pathogen, even if the host has never been exposed. The ubiquitous presence of Nabs in common carp has been demonstrated using indirect ELISA. Variation in serum levels of Nabs in different age groups, environmental conditions and genetic background has been noticed. Thus the level of natural antibodies can also serve as a marker for selection for disease resistance, as discussed by Kachamakova *et al.*[18].

The present investigation was undertaken to determine the genetic variation in non-specific immune parameters from different fullsib families of Indian major carp, *L. rohita* and to study the genetic associations between these parameters and survival against challenge with *E. tarda*. Rohu (*L. rohita*) is one of the most important Indian major carp species used in small-scale and intensive fish farming.

Materials and methods

Fish

Rohu (24.26 ± 0.77 g) was obtained from the fourth generation of the fish selected for increased growth in an Indo-Norwegian collaborative project carried out at the

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Central Institute of Freshwater Aquaculture (CIFA), Bhubaneswar, India[19]. The fullsib families were produced using a nested mating design with an average of two dams per sire (Figure 1) and brought up in equal-sized earthen ponds to advance fingerling stage. Individual families were stocked in 700 l ferro-cement tanks in a wet laboratory. The fish were fed with a standard pelleted diet at 3% of their body weight in two divided doses daily during the experiment. Water of the tanks was exchanged partially daily to remove waste feed and faecal material.

Experimental design

The fish were shifted to 500 l capacity FRP tanks from the stock tank for conducting different experiments. They were acclimated for 15 days in the tanks before conducting any assay. Fish from each family were distributed into five tanks with 10 fish each. The fish in three tanks were utilized for challenge study and those in the other two tanks for collection of blood. Water temperature in the tanks varied from 28 to 30°C during the experiment.

Sampling

Fish were bled through caudal vein after anesthetizing them with MS 222 (Argent Chemical, Redmond, USA). An aliquot of the blood was heparinized (50 IU ml^{-1}) and the remaining part was allowed to clot at room temperature and then kept at 4°C. The collected serum samples were stored at –70°C till further analysis for various serum parameters.

Non-specific immune responses

Reactive oxygen radical production by neutrophils during respiratory burst activity was assayed by the reduction of nitroblue tetrazolium (NBT) to formazan[20]. Briefly, blood was mixed with 0.2% NBT in equal proportions (1 : 1) and incubated for 30 min at 25°C. Fifty microlitres of this mixture was taken out and 1 ml of dimethyl formamide (SRL, India) was added to solubilize the reduced formazan product. Then it was centrifuged at 2000 *g* for 5 min and the supernatant was taken. The extent of NBT reduced was measured at an optical density of 540 nm using dimethyl formamide as the blank.

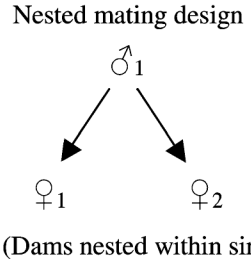

Figure 1. Mating design for production of fullsib families of rohu (*Labeo rohita*).

The total myeloperoxidase content of serum was determined as described by Quade and Roth[21], and partially modified by Sahoo *et al.*[22]. Briefly, 10 µl of serum was diluted with 90 µl of HBSS without Ca^{2+} or Mg^{2+} in 96-well microtitre plate to which 35 µl of 20 mM 3,3′,5,5′-tetramethyl benzidine hydrochloride (Genei, India) and 5 mM H_2O_2 were added. After 2 min of incubation, 35 µl of 4 M sulphuric acid was added to stop the reaction. Optical density was read at 450 nm in a microtitre plate reader (Anthos 2010, Austria).

The lysozyme content of serum was determined by turbidimetric assay according to Sankaran and Gurnani[23], with partial modifications. A suspension of 150 µl of *Micrococcus lysodeikticus* (0.2 mg ml^{-1} in 0.02 M sodium acetate buffer, pH 5.5) was added to previously dispensed test serum (15 µl) in a 96-well U-bottom microtitre plate and initial OD was taken at 450 nm immediately. The final OD was taken 1 h after incubation[22] at 24°C. A standard curve was prepared using lyophilized hen egg white lysozyme (HEWL; Sigma, USA). Serum lysozyme values were expressed as µg/ml equivalent to HEWL activity.

Haemagglutination assay (HA) was performed as described by Kumari and Sahoo[24]. Double serial dilution of the inactivated sera (56°C for 20 min) was made in PBS (with Ca^{2+} and Mg^{2+}), and then 50 µl of 1% rabbit RBC (RaRBC) was added to each well of the microtitre plate and incubated for 1 h at 37°C. The HA titre was defined as the last dilution of serum showing minimal positive agglutinin. Values are expressed as reciprocal of HA titre. The haemolytic ability of unheated serum was also determined by diluting similarly with PBS (without Ca^{2+} and Mg^{2+}) and using RaRBC. Plasma glucose content was quantified by enzymatic colorimetric method with GLUCOSE FL kit (Chema Diagnostica, Italy).

Natural agglutinin levels in the serum of individual fish were determined by plate agglutination technique[25]. Briefly, inactivated sera were diluted twofold serially in PBS (with Ca^{2+} and Mg^{2+}) in a 96-well microtitre plate and then 50 µl of formalin-killed *E. tarda* (adjusted to Mac Farland's Standard No. 9) was added to each well. The bacterial agglutination titre was defined as the last dilution of serum showing minimal positive agglutinin. Values were expressed as reciprocal of the agglutination titre.

Challenge test

The fish were challenged with a local isolate of virulent *E. tarda* (ImEt1/03) maintained at the Aquatic Animal Health Division, CIFA to find out the susceptibility pattern of different families. Three tanks with ten fish each for a family were utilized for the challenge study. The bacterial isolate was grown in tryptone soya broth at 30°C for 23 h. The LD_{80} dose was calculated using a fish population of mixed families with equal number of individuals from each family, following Reed and Muench[26]. Fish were injected intraperitoneally with LD_{80} dose of 3.5×10^7 live

Table 1. Mean values and standard errors for each trait of different families of rohu

Family no.	Lysozyme ($\mu g\ ml^{-1}$)	Myeloperoxidase (OD at 450 nm)	Nitroblue tetrazolium assay (OD at 540 nm)	Glucose ($mg\ dl^{-1}$)	Haemolysin titre	Haemagglutination titre	Bacterial agglutination titre	Per cent survival of fish
5	5.14 ± 0.90^{def}	0.99 ± 0.10^{ab}	0.44 ± 0.04^{bcd}	34.45 ± 2.82^{a}	1.20 ± 0.13^{a}	3.35 ± 0.64^{a}	38.67 ± 4.67^{abc}	50.83 ± 14.15^{d}
7	6.95 ± 0.91^{fg}	0.80 ± 0.10^{a}	0.34 ± 0.01^{ab}	63.33 ± 5.44^{cd}	1.38 ± 0.15^{ab}	8.80 ± 1.91^{a}	41.33 ± 4.04^{abc}	19.44 ± 8.47^{abcd}
9	8.89 ± 1.05^{g}	1.12 ± 0.09^{b}	0.46 ± 0.04^{bcd}	55.98 ± 5.27^{bc}	1.29 ± 0.13^{a}	2.90 ± 0.64^{a}	54.00 ± 9.93^{c}	50.00 ± 12.65^{cd}
14	6.51 ± 0.75^{ef}	0.99 ± 0.12^{ab}	0.37 ± 0.05^{abc}	82.53 ± 8.49^{de}	3.13 ± 0.66^{c}	6.00 ± 1.78^{a}	27.40 ± 5.30^{ab}	23.65 ± 7.32^{abcd}
15	5.13 ± 1.00^{def}	1.00 ± 0.09^{ab}	0.46 ± 0.05^{bcd}	86.59 ± 4.90^{ef}	2.60 ± 0.65^{abc}	3.20 ± 0.66^{a}	41.60 ± 6.40^{abc}	44.62 ± 12.90^{bcd}
19	1.04 ± 0.17^{ab}	1.57 ± 0.06^{d}	0.49 ± 0.04^{bcde}	54.55 ± 4.15^{bc}	1.60 ± 0.40^{abc}	4.70 ± 0.79^{a}	20.40 ± 3.98^{a}	6.67 ± 3.65^{abc}
20	4.29 ± 0.64^{cde}	1.63 ± 0.09^{d}	0.39 ± 0.03^{abc}	109.98 ± 13.82^{gh}	2.00 ± 0.37^{abc}	2.60 ± 0.40^{a}	21.33 ± 2.35^{a}	25.00 ± 0.00^{abcd}
28	6.41 ± 0.93^{ef}	1.47 ± 0.09^{d}	0.37 ± 0.03^{abc}	106.53 ± 4.45^{g}	1.70 ± 0.30^{abc}	3.13 ± 1.48^{a}	32.00 ± 0.00^{abc}	30.00 ± 9.49^{abcd}
31	3.29 ± 0.53^{bcd}	1.49 ± 0.08^{d}	0.33 ± 0.01^{ab}	54.14 ± 2.56^{bc}	1.50 ± 0.17^{abc}	2.10 ± 0.72^{a}	18.00 ± 2.78^{a}	0.00 ± 0.00^{a}
32	2.49 ± 0.52^{abc}	1.20 ± 0.08^{bc}	0.52 ± 0.05^{cde}	127.40 ± 4.55^{h}	1.60 ± 0.16^{abc}	10.20 ± 6.16^{a}	38.40 ± 7.52^{abc}	100.00 ± 0.00^{e}
43	1.66 ± 0.26^{ab}	1.17 ± 0.06^{bc}	0.63 ± 0.10^{e}	77.16 ± 7.47^{de}	3.04 ± 0.65^{bc}	3.38 ± 0.86^{a}	96.17 ± 21.81^{d}	20.00 ± 3.16^{abcd}
45	3.29 ± 0.51^{bcd}	1.11 ± 0.07^{b}	0.40 ± 0.04^{abc}	78.67 ± 7.91^{de}	1.67 ± 0.12^{abc}	31.40 ± 9.02^{b}	26.00 ± 5.50^{a}	0.00 ± 0.00^{a}
52	0.76 ± 0.13^{a}	0.94 ± 0.11^{ab}	0.56 ± 0.07^{de}	103.00 ± 5.88^{fg}	1.20 ± 0.13^{a}	2.00 ± 0.37^{a}	20.00 ± 5.37^{a}	37.50 ± 0.00^{abcd}
64	8.78 ± 1.31^{g}	1.38 ± 0.08^{cd}	0.34 ± 0.02^{ab}	72.73 ± 2.06^{cde}	5.40 ± 1.39^{d}	9.90 ± 2.91^{a}	22.50 ± 3.27^{a}	0.00 ± 0.00^{a}
70	2.91 ± 0.37^{abcd}	1.41 ± 0.05^{cd}	0.27 ± 0.04^{a}	42.51 ± 4.39^{ab}	2.80 ± 0.42^{abc}	4.60 ± 0.79^{a}	52.00 ± 10.75^{bc}	3.33 ± 1.83^{ab}

Means bearing common superscript(s) in the column did not differ significantly ($P < 0.05$) between families.

Table 2. Correlation coefficients for Spearman's rank order correlation

Parameter	Per cent survival of fish
Lysozyme ($\mu g\ ml^{-1}$)	0.054
Myeloperoxidase (OD at 450 nm)	–0.319
Nitroblue tetrazolium assay (OD at 540 nm)	0.556*
Glucose ($mg\ dl^{-1}$)	0.366
Haemolysin titre	–0.397
Haemagglutination titre	–0.280
Bacterial agglutination titre	0.348

*Correlation is significant at the 0.05 level (two-tailed).

cells of *E. tarda* per g body weight. The fish were watched for 10 days for mortality and per cent survival calculated. The cause of mortality was confirmed by reisolating the bacteria from the kidney of 10% dead fish, as described by Kumari *et al.*[27].

Statistical analysis

Experimental assays were performed in duplicate for each parameter of the individual fish of the family and the mean ± SE for each family was calculated. Mean values were compared by one-way ANOVA followed by Duncan's multiple range test[28] to determine significant difference at 5% ($P < 0.05$) level. Spearman's rank order correlation was used to find any correlation between survival and immune parameters.

Results

Non-specific immune responses

A wide variation in the immune parameters with significant differences among the families of rohu was observed (Table 1). Lysozyme levels of individual rohu ranged from 0.08 to 16.62 $\mu g\ ml^{-1}$ of serum, with mean value of 4.5 $\mu g\ ml^{-1}$. The myeloperoxidase activity of serum varied from 0.23 to 1.96, with mean value of 1.22. NBT activity in serum ranged from 0.13 to 1.15 with mean value of 0.42. The amount of glucose present in blood ranged from 21.14 to 202.35 $mg\ dl^{-1}$, with a mean value of 76.64 $mg\ dl^{-1}$. Titres for haemolysin, haemagglutination and bacterial agglutination varied from 1 to 16, 1 to 64 and 1 to 256, with their respective mean values of 2.14, 6.55 and 36.65.

Challenge test

A large variation in the susceptibility pattern among different families of rohu to *E. tarda* infection was also observed. The per cent survival ranged from 0 to 100, with mean value of 27.4 against *E. tarda* challenge.

Table 2 shows correlation coefficients between the least square means of 15 families for the different immune parameters, and survival from *E. tarda* infection. A significant positive ($P < 0.05$) correlation between NBT activity and survival was detected. On the other hand, there was no significant correlation between the other immune parameters and survival against *E. tarda* challenge.

Discussion

Variation in innate immune response and survival against *E. tarda* challenge was examined for fifteen fullsib families of rohu. The fish were obtained from the fourth generation of on-going selection programme of rohu for growth, for which fish had been collected from a highly diverged riverine system of India. Although the fish were selected for growth, they exhibited wide variation in disease resistance and immune response.

Lysozyme is found in fish mucus, serum and ova[29], which helps in degrading peptidoglycan layer of the cell wall of Gram-positive and Gram-negative bacteria after lysis of the outer lipopolysaccharide layer by the action of complement[30]. It also promotes phagocytosis as an opsonin, or by directly activating polymorphonuclear leucocytes and macrophages. The relatively large variation observed in the levels of lysozyme in different families reflects the variation in their ability to destroy Gram-negative bacteria. Such variation in serum lysozyme levels was also observed in the families of Atlantic salmon and rainbow trout by Roed *et al.*[31] and Roed *et al.*[15] respectively, selected for disease resistance. Production of reactive oxygen species such as superoxide anion (O_2^-), and hydrogen peroxide by fish macrophages and neutrophils is involved in destroying pathogens[32]. Neutrophils also contain a large amount of myeloperoxidase, which is involved in the production of bactericidal hypohalites. The rohu families showed significant differences in NBT and myeloperoxidase activities, suggesting variation in oxygen-dependent mechanisms to kill pathogens. Blood glucose levels are increased in fish following exposure to stressful stimuli. This is due to rapid increase in catecholamines followed by cortical-dependent gluconeogenesis[33]. There exists inherent differences in blood glucose levels within different genetic populations in a species, and higher or lower blood glucose levels have been correlated with disease susceptibility. Thus, the existence of wide variation in plasma glucose levels suggests that some of the families may adapt better to stressful conditions, as during infection, compared to others. Fish serum is capable of causing haemolysis of heterologous red blood cells. Such haemolytic activity of blood serum of fish is the result of classical and alternative pathways of the complement system as in the case of mammals. Differences in haemolysin titres observed in different families are in agreement with the findings of other workers[17,34,35]. Agglutinins are a group of proteins, which have different specificity for carbohydrate binding[36]. These agglutinins are Ca^{2+}-dependent and can agglutinate a number of fish bacterial pathogens. These probably bind to the carbohydrate moieties on the surface of bacteria, which are involved in attachment to the integumental cells, thus blocking attachment and subsequent invasion of the host. The natural agglutinin levels in serum were estimated by haemagglutination and bacterial agglutination assays. Though significant variation for bacterial agglutination titre was observed among the families, there was not much variation in haemagglutination titre.

Genetic variation in fish strains for resistance against specific pathogens at the inter- and intra-specific levels has been observed by earlier workers[7,8,10–12,15,35,37–40]. The mortality range of 0–100% against edwardsiellosis shows wide variation in susceptibility in various families of rohu as demonstrated against *A. hydrophila* infection in Atlantic salmon (*Salmo salar*)[8] and rohu[12]; viral haemorrhagic septicaemia in rainbow trout[10]; *G. salaris* infection in Atlantic salmon and rainbow trout[13]; koi herpes virus in common carp (*Cyprinus carpio* L.)[14], and *Lepeophtheirus salmonis* in Atlantic salmon (*S. salar*)[41]. This suggests that there is enormous scope for direct selection for disease resistance in rohu.

Earlier workers have shown that significant correlation exists between disease resistance of fish and non-specific immune parameters, viz. serum lysozyme, complement, haemolytic and bactericidal activities, which affect the inherent capacity of fish to resist pathogens prior to generation of a specific immune response[8,11,12,15,31,34,42,43]. For example, a linkage between resistance to furunculosis, and low lysozyme activity, low antibody titres against *Vibrio salmonicida* O-antigen and high IgM levels in Atlantic salmon has been noticed[7]. Positive correlation between superoxide production during respiratory burst activity and survival against *E. tarda* challenge was noticed in this experiment, as observed by Balfry *et al.*[16] in coho salmon and tilapia against *Vibrio* sp. Though serum lysozyme and myeloperoxidase levels, haemolysin and haemagglutination titres against RaRBC, bacterial agglutination titre and blood glucose content did not show any significant correlation with the survival against edwardsiellosis, their potential as useful marker traits for indirect selection to improve disease resistance, as noted by earlier workers, cannot be overlooked. Lund *et al.*[7] and Chiayvareesajja *et al.*[44] also reported that there was no significant correlation between haemolytic activity and survival rate in Atlantic salmon and Nile tilapia respectively, against various bacterial pathogens. This suggests that serum haemolytic activity is a relatively poor marker trait for indirect selection for disease resistance in rohu[12].

This preliminary work on the study of variation of immune responses in different families of rohu provides ample information for further investigations in this field, with larger sample size to establish more marker traits for resistance to edwardsiellosis, and possibly against other pathogens as well.

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ACKNOWLEDGEMENTS. We thank the project personnel of the Indo-Norwegian Project at CIFA for help during the study. Thanks are also due to the Director, CIFA for providing necessary facilities during this study.

Received 11 July 2006; revised accepted 23 January 2007