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Tissue specific expression profile of some immune related genes in *Labeo rohita* to *Edwardsiella tarda* infection

Sajal Kole [a, 1], Deepika Anand [a], Rupam Sharma [b], Gayatri Tripathi [a], M Makesh [a], KV Rajendran [a], Megha Kadam Bedekar [a, *]

[a] Aquatic Environment and Health Management Division, ICAR- Central Institute of Fisheries Education, Mumbai 400061, India
[b] Fish Genetics and Biotechnology Division, ICAR- Central Institute of Fisheries Education, Mumbai 400061, India

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ABSTRACT

Rohu (*Labeo rohita*), an Indian Major Carp (IMC) is an economically important aquaculture species in India. Inspite of the technological advances, infectious diseases caused by viruses, bacteria and parasites have been a major limiting factor in the development and profitability of fish farms. At present, information regarding the immune status of the Indian major carps is limited. This lack of knowledge is a major impediment for establishment of effective preventive measures against broad spectrum of infectious agents. The present study was undertaken to examine the modulation of few immune-regulatory genes: IgHC, NOD 1, TLR 22, iNOS and IL-1β during experimental infection of *E. tarda* in *L. rohita* to understand their role in pathogenesis. Rohu fingerlings were intra-peritoneally injected with *Edwardsiella tarda* (LD_{50} dose of 8.7×10^4 CFU/fish) and sampled for three immunologically important organs (kidney, liver and spleen) at different time intervals (zero hour or pre-challenge and 6 h, 12 h, 24 h, 48 h and 96 h post challenge). For absolute quantification of genes by real time RT-PCR, all the genes transcript were amplified from Poly I:C induced rohu lymphocytes and cloned in pTZ57R/T plasmid. Standard curves for each gene was generated from serially diluted plasmid bearing respective genes. Evaluation of copy number of different genes present in the tissue showed that the expression of IgHC, iNOS and IL-1β was highest in kidney followed by spleen and least in liver. While for NOD 1 and TLR 22 gene, liver showed higher expression than kidney and spleen. Further, the expression of IgHC, INOS, TLR 22, NOD 1 and IL-1β genes significantly differed ($P < 0.05$) in the *E. tarda* challenged fish when compared with pre-challenged control fish. Among the five genes we studied, the basal expression of TLR 22 gene was highest. The result also depicts that iNOS and NOD 1 are immediate responsive genes as their expression reached maximum level at 6–24 h post infection (hpi) after which the expression declined. In contrast, TLR 22 and IgHC gene transcript showed enhanced expression during the late phase of with maximum expression observed after 48 hpi and 96 hpi respectively. IL-1β, being the exception, showed high expression both at 24 hpi and 96 hpi. From this study, we conclude that these five immune genes have a definite role to play in the defense mechanism of host (*L. rohita*) against *E. tarda*.

1. Introduction

Fish farming in India is mainly centered on carp culture which accounts for nearly 80% of total freshwater fish production. Among the large number of potential cultivable carp species, three Indian Major Carps (IMCs) i.e. catla (*Catla catla*), rohu (*Labeo rohita*) and mrigal (*Cirrhinus mrigala*), contribute over two million tonnes [1]. Rohu is the most preferred species because of its high growth potential, consumer preference and compatibility with other carps in polyculture system. However, the intensification of carp culture causes frequent occurrence of infectious diseases which lead to massive economic loss in aquaculture production. One of most prevalent bacterial diseases in India, causing economic losses to the aquaculture industry, is edwardsiellosis caused by *Edwardsiella tarda* leading to putrefactive systemic infection and mass mortality in various populations and age groups of fishes [2].

* Corresponding author.
E-mail addresses: sajal.kole@gmail.com (S. Kole), megha.bedekar@cife.edu.in (M. Kadam Bedekar).
[1] Present Address: Department of Aqualife Medicine, Chonnam National University, Republic of Korea.

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E. tarda, a Gram negative bacterium, has been reported to infect a wide range of fish like channel catfish, Japanese eel, Japanese flounder, tilapia, carp, largemouth bass, chinook salmon and rainbow trout. The enormous loss caused by this pathogen is being observed in the USA, Japan, Europe and Asian countries; although, no exact figures are available to quantify the loss. The disease prevalence in ponds is seldom above 5%; however, it can reach 50% when fish are confined to tanks [3]. Experimental and natural infections by *E. tarda* in Indian major carps have also been reported [4–6]. The pathology related to the disease in fish includes the presence of distended abdomen, prolapsed rectum, cutaneous lesions inside the musculature, fibrinous peritonitis, presence of gas pockets in the kidney or musculature and also necrosis of the hepatic and renal tissue [7]. Although introduction of vaccines has greatly reduced the traditional antibiotic mode of control, the limited knowledge on immune system of fish is a major impediment in the development of new vaccines based on non-empirical strategies [8]. Furthermore, how the bacterium modulates the host immune response to its advantage as well as the factors that contribute to the pathogenesis has been poorly understood. Therefore, the role of various immune-regulatory genes on bacterial infection needs to be resolved to develop a novel immunization strategy to cater the disease problem.

Bacterial infection results in a coordinated activation of immune responses in the host. Nucleic acids of the microorganisms and conserved pathogen derived structures like peptidoglycan (PGN), lipoteichoic acid (LTA), zymosan, flagellin, lipopolysaccharides (LPS), lipoproteins, heat shock protein (hsp), CpG-DNA which are pathogen/microbes-associated molecular patterns (PAMPs/MAMPs), induce host pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) and nucleotide binding and oligomerization domain -like receptors (NLRs) to initiate an innate immune response [9]. TLR-associated signal-transduction pathways have been identified as crucial components of innate immunity, found in all invertebrates and vertebrate species examined to-date. Among various types of TLRs, TLR 22 is exclusively present in teleost in functional form, is reported to be up-regulated with respect to gram negative bacteria or viral (simulated with poly I:C) infections [10–12]. This receptor senses bacterial RNA and induces activation of inflammatory factors to create a non-specific immune response by enhancing production of cytokines and control bacterial growth [13]. Nucleotide binding and oligomerization domain-1 (NOD1) is a cytoplasmic PRR, and is a member of the NOD-like receptor (NLR) family. A wide range of bacteria and viruses have been reported to be detected by NOD-1 receptor [14]. It has also been shown to activate both type I and type II interferon creating an anti-microbial environment in the host [15,16].

The innate immune system involves both fixed and mobile cells, and large number of molecules dissolved in body fluids. Cytokines like interleukin 1β (IL-1β) is one of the pivotal early response pro-inflammatory cytokines that enables organisms to respond to infectious insults, inducing an inflammatory cascade, along with other defensive responses [7]. It is a key mediator in response to microbial invasion and tissue injury and can stimulate immune responses by activating lymphocytes or by inducing the release of other cytokines capable of triggering macrophages, Natural Killer (NK) cells and lymphocytes [17]. These cytokines bind to iron and create a bacteriostatic environment by limiting the availability of iron to replicating pathogens. It also induces other gene products such as lysozyme, protease inhibitors, and complement proteins which may play a role in further restricting their multiplication or pathogenesis [18,19]. Nitric oxide, a multifunctional effector molecule synthesized by nitric oxide synthase (NOS) from L-arginine, conveys signals for vasorelaxation, neurotransmission, and cytotoxicity. The inducible NOS (iNOS), a multifunctional effector molecule and a well known immune-regulatory factor important in the defence mechanism against various pathogens have been studied previously [20–22]. Henceforth, the importance of iNOS in killing of bacteria needs further study.

Resistance to microbial infection by host also involves specific humoral factors i.e. antibody response. It is the third line of defense mechanism. IgM is known to be an indicator of specific immune responses and is the major immunoglobulin (Ig) isotype of teleost fish [23]. Teleost fish utilize a unique pattern of RNA processing to generate the secreted and membrane receptor forms of the IgM heavy chain [24]. Thus, the role of IgHC is critical in disease resistance and pathogenesis due to bacterial infections.

The present study was conducted to evaluate the modulation of immune-related genes viz, IgHC, NOD 1, TLR 22, iNOS and IL-1β in *L. rohita* in response to experimental infection with *E. tarda* in order to have a better understanding of the response of these genes in pathogenesis. The variation in the expression kinetics of the genes at different time intervals post-infection with the bacterium in three immunologically important organs viz. kidney, liver and spleen are discussed.

2. Material and methods

2.1. Fish

Rohu (*L. rohita*) juveniles (25.0 ± 2.6 g) collected from a local farm were stocked into 500 L fiber reinforced plastic (FRP) tanks with aerated freshwater in our indoor rearing facility for 3-weeks prior to infection and were fed twice a day with a standard pelleted diet at 3% of their body weight. Water quality of the tanks was maintained. The water temperature varied from 27 °C to 28 °C and the pH of the water varied from 7.4 to 7.6 during the experiment. To confirm the fishes were *E. tarda*-free, bacterial isolation in SS-agar plate from kidney and liver of ten randomly selected individuals was carried out. After confirmation, the fishes were distributed into two groups (n = 40/group), one for time oriented sampling post infection and the other for observation of mortality pattern after challenge.

2.2. Bacteria

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 Salmonella-Shigella Agar (SS agar) media and incubated at 28 °C. Single black colony grown on agar plate was inoculated in BHI broth and incubated at 28 °C for 22 h.

2.3. In vivo bacterial challenge

Viable count of *E. tarda* 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 [25] from same experimental group of fishes (10 fish per dilution). For experimental infection, 40 fish per group were intraperitoneally injected 100 μL of bacterial suspension (LD_{50} dose of 8.7×10^4 CFU/fish). For immune-related gene expression study, time-oriented sampling was conducted at pre-infection (zero hour) and 6, 12, 24, 48 and 96 h post infection (hpi) from one group. Three fish were randomly selected at each time point. The samples collected at pre-challenge were taken as control. The kidney, liver and spleen tissues of fish were aseptically dissected and collected separately in TRIzol® reagent (Invitrogen, USA) and used for RNA isolation. In the other group mortality pattern was observed for 14