Introduction

Isolation and characterization of immune responsive genes using transcriptome profiling of bacterial challenged Golden Mahseer
Chapter 1

Introduction

Fishes represent an important group of organism in the animal kingdom. In fact, with the largest number of species (estimated number 33,200) among vertebrates, they exhibit greater species diversity than any other group (http://www.fishbase.org/). Fishes are also an inseparable food resource with the world’s largest extractive use of wildlife resources (FAO, 2012). According to FAO’s (2012; 2014) estimates, fishes accounted for 16.7 % of the World’s population intake of animal protein and 6.5 % of all protein consumed. Fisheries and aquaculture also plays an important role in World’s economy, trade, nutritional security and livelihood to substantial population. As per the FAO (2014) report, fisheries sector provides jobs to 10 million people and support the livelihood of nearly 100 million people Worldwide. Concisely, aquaculture is an important food as well as recreation resource.

Fisheries and aquaculture are important areas of food production and employment in India and other developing countries, where fisheries contributes the half worth of their traded food commodities (NFDB, 2016). India is the world’s 7th largest producer in marine capture fisheries (3.4 million tonnes) while second largest in inland water capture (1.4 million tonnes) and farmed food fish production (4.2 million tonnes) (FAO, 2014). Initially during the years 1950-2010, the fish production was more from marine capture fisheries as compared to culture fish production (NFDB, 2016). The marine capture fishery may have reached to saturation and since last one decade no significant increment was reported, while culture fishery is having potential of achieving more production (FAO, 2014). It is also apparent that the culture fishery is contributing more than the marine capture and inland water capture fisheries of Indian fisheries resources (NFDB, 2016). Among the total aquaculture production in India, about 95% is contributed by freshwater aquaculture (FAO, 2014).

Freshwater aquaculture comprises the culture of carp species, catfishes, freshwater prawn, and few other species, while in the upland Coldwater region trout and mahseer farming has significant potential for aqua farming (FAO, 2014). Despite the possibilities of aquaculture production, there is not much expansion in fisheries in cold water regions (Himalayan region) but the contribution of cold water fisheries with indigenous (mahseer and schizothoracids) and exotic (trouts) species is also of great impotence (FAO, 2014). The expansion, intensification and diversification of
aquaculture had also favoured the spread of pathogens and diseases into aquaculture systems (Walker and Winton, 2010). Along with the increased production, aquaculture systems of the country must also focus on water quality monitoring and fish health management. Fish health management is equally important along with improved aquaculture practices (FAO, 2014). Also, we need to look beyond the economic gains and focus on responsible and sustainable fisheries as well as ensure the healthy food production.

Diseases are one of the primary constraint on aquaculture growth and responsible for the severe economic losses in many countries of the world (Pillay and Kutty, 2005; Leung and Bates, 2013). Addressing the fish health issues, preventive and remedial programmes have become an urgent requirement for sustainable aquaculture (Leung and Bates, 2013). Studies are now more focused on prophylactic measures including vaccination, probiotics and immune-stimulation in fish health management (Magnadottir, 2010). The major advantage of stimulated immune control of fish disease is the increase in fish defence system to multiple pathogens simultaneously. Research are now more targeted on the use of molecular tools in the development of prophylactic measures and their integration in breeding programs for disease resistance (Magnadottir, 2010).

Fishes are also considered the best model organism to study the evolution of the immune system (Zhu et al., 2013). They possess an efficient immune system to fight against the diseases and invading pathogens, but it is not as developed as in mammals. Also, their habitat is more prone to pathogenesis compared to other vertebrate organisms. Fish immune system is summarized either using few model fish species like Danio rerio, Oryzias latipes, Takifugu rubripes or by comparing it to higher vertebrate and mammalian immune system (Flajnik and Kasahara, 2010). The immune function of other cyprinids may have similarities with model fishes, but the diagnostic and disease preventive measures may not be equally applicable to the entire taxa. Also, all the higher vertebrates possess much evolved and functional immune organ homologues of fishes (Rauta et al., 2012). There are lots of queries about the exact functioning of the fish immune system and its interaction after the pathogen invasion. So deciphering the immune system of the model as well as non-model fish species to understand its evolution, function as well as formulating preventive measures has gained lots of attention in research (Van Muiswinkel, 2008; Lieschke and Trede, 2009, Zhu et al., 2013).
Figure 1.1: An overview of the fish immune system.
The function of the entire fish immune system is much complex and beyond the context of the present study so we are only restricting to the immune system of cyprinids and their defence mechanism in bacterial diseases. The immune components and pathways involved in fish defence in viral pathogenesis are also not addressed in the present study. Figure 1.1 summarizes the basic components of the fish immune system their interaction during pathogenesis.

Widely the immune system of fishes is divided into innate and adaptive immune system; furthermore, these two comprises cellular components and humoral factors that function together (both innate and adaptive components) during pathogenesis to eliminate the invaders (Tort et al., 2003). Innate immunity is the first line of defence that includes all the components present in organism since birth and even before the encounter of any pathogenic agent. They are nonspecific in function, and a common action is initiated against varied antigenic agents regardless their form and severity (Uribe et al., 2011). The innate immune system comprises physical barriers (skin, scales, mucous layer, etc.), cellular components (granulocytes, monocytes, macrophages and natural killers cells) and humoral factors like antimicrobial enzymes, interleukins, interferon, etc. (Bayne and Gerwick, 2001; Magnadottir et al., 2011; Biller-Takahashi and Urbinati, 2014). The cellular and humoral components may interact to initiate an inflammatory response which is also considered an innate immune response (Uribe et al., 2011).

Physical protections like integumentary system (scales, dermis and epidermis) serves as the primary defence which is also aided by bactericidal and fungicidal mucus covering (Shephard, 1994; Ellis, 2001). A physical defence mechanism is also available in various tissues that are main targets of invasion. As soon as the pathogen invasion is detected, the body coordinates various efforts to resist the pathogen entry paths. Histamine and other pro-inflammatory proteins are also secreted by damaged cells that cause inflammation and narrow down the blood cells. At the same time fibrinogen and other clotting factors interact and creates a physical barrier of fibrin (Tavares-Dias and Oliveira, 2009). Alongside various innate and adaptive components also get activated and coordinate to encounter the invaded pathogens. The nonspecific defences act immediately after infections, whereas specific immunity function to overcome the pathogenesis after the invasion (Uribe et al., 2011).

The components of innate immune system mainly recognize ‘pathogen-
associated molecular patterns (PAMPs) which are mainly the signature molecules like lipopolysaccharide (LPS) and peptidoglycan (PG) of bacterial cell wall, bacterial DNA or viral RNA, or other ‘non-self’ molecules generally found in membranes of multicellular organisms with the help of their pattern recognition receptors (PRR) (Lee and Kim, 2007; Biller-Takahashi and Urbinati, 2014). The granulocytes are first cells to arrive at the inflammation site and initiate the pathogen destruction while the other phagocytic cells like macrophages engulf the bigger pathogenic components and cellular debris at the site of infection (Goldsby et al., 2002; Elward and Gasque, 2003; Magnadottir, 2006; Boltan et al., 2011).

The complement system is also an integral part of a fish innate immune system that comprised of nearly 35 soluble and membrane-bound proteins (Boshra et al., 2006). The fish complement component’s diversity is more than that of mammals where the C3 component has shown to comprise at least five isoforms in a single species (Holland and Lambris, 2002). Although complement cascade is a multifunctional system, it majorly acts through membrane attack complex (MAC) to kill pathogens.

Though, fish immune system was considered to comprise majorly innate immune system, recent studies revealed the presence of adaptive immune components in fishes (Stockhammer et al., 2009). The adaptive immune system mainly functions after the recognition of pathogen-specific antigens. These antigens are recognized either by specific antibodies or the antigen presenting cells (APC) such as macrophages, dendritic cells and B lymphocytes along with generating the immune memory against the same antigen for the second encounter (Bernstein et al., 1998). These antigen presenting cells primarily comprises the receptor for T lymphocytes and help in antigen recognition (Lewis et al., 2014). T-cells recognize these antigens only in the presence of specific humoral glycoprotein receptors (present on APC) known as major histocompatibility complex (MHC) (Goldsby et al., 2002; Laing and Hansen, 2011). After the recognition of antigens, these T cells secretes cytokines that further regulate and accumulate B lymphocytes, cytotoxic lymphocytes, macrophages and other immune cells to destroy the invading pathogen (Abbas and Lichman, 2004, Salinas et al., 2011). Like the complement components, antibodies when bind to specific antigens may directly initiate its neutralization or opsonization (Sakai, 1984). Cytotoxic T lymphocytes works on larger antigens and where antigenic molecules are intracellular (Biller-Takahashi and Urbinati, 2014).
All the mechanisms work in a cascade and interactive manner and each function either get stimulated by preceded one or initiate the proceeding pathway. However tracking each and every pathway or regulatory molecule to elucidate their ultimate/significant function in any biological process or disease is not very much feasible. Hence, the ‘omics’ approaches are most suitable to study the overall system biology in a multi-dimensional manner (Horgan and Kenny, 2011). These holistic strategies have many applications and can be applied to normal physiological processes as well as in disease conditions. These studies aid our understanding in screening, diagnosis, prognosis and etiology of diseases (Horgan and Kenny, 2011).

Transcriptome profiling (or transcriptomics) uses deep-sequencing technologies to study the functional elements of the genome and reveal the major constituents of cells and tissues working during a biological process (Wang et al., 2009a). The major advantage of RNA-Seq technology is its efficiency to detect novel molecules and does not require any prior information about the genes (Qian et al., 2014). This makes transcriptome sequencing (RNA-Seq) attractive particularly for non-model organisms whose genomic sequences are yet not available (Wang et al., 2009a). This approach can be exploited to understand the variation in gene expression level in a test sample by comparing the expression level of the same gene in a control. It is also useful in finding the novel genes linked to the diseased condition by tracking the entire transcriptome. The advancement of bioinformatics approaches aids the RNA-Seq technology and also helps in determination of the ultimate pathways which are regulated by the putative functional regulators (Qian et al., 2014). Both RNA-Seq and bioinformatics techniques together provides an opportunity to study the immune system of species in a comparative manner (Schunter et al., 2014). The preventive measures formulated using these specific studies are surely more applicable instead of adopting them from congeneric species or model organisms.

Mahseers, belong to the family Cyprinidae, are well known for the excellent game as well as food fishes all over the world especially the South-Asian countries (Mohindra et al., 2007; Naeem et al., 2011; Hussain, 2012; Shahi et al., 2014; Bhatt and Pandit, 2016). In India, eight mahseer species are available among which Golden Mahseer, *Tor putitora* is considered the candidate species for hill aquaculture in the mid-Himalayan range (Bhatt and Pandit, 2016). The artificial breeding, seed production and scientific management of Golden Mahseer are in high priorities (Pandey et al., 1998; Bhatt et al., 2004; Shahi et al., 2014). Scientific management
including water quality, feed and health management are also equally important for sustainable aquaculture (Pillay and Kutty, 2005; Leung and Bates, 2013). But, the data regarding the health management, mainly diseases is least available for this important fish species (Shahi et al., 2014; Barat et al., 2015). Also, there is no diagnosis, preventive and treatment measures available so far for the species. There are only a few reports on microbial diseases in the fish that have been reported from natural stocks (Mallik et al., 2010; Shahi and Mallik, 2014). Along with its large scale production and domestication, remedial measures need to be developed simultaneously to prevent any possible economic losses in future.

Aquaculture is primarily affected by microbial pathogens especially of bacterial origin (Zorrilla et al., 2003), wherein, *Aeromonas hydrophila* and other *Aeromonas* are responsible for the majority of diseases (Janda and Abbott, 2010). *Aeromonas hydrophila* and other congeneric species are among the major mesophilic pathogenic candidates of freshwater fishes (Hazen et al., 1978; Janda and Abbott, 2010; Tomas, 2012; Beaz-Hidalgo and Figueras, 2013). *A. hydrophila* is reported being primary bacterial pathogen affecting freshwater fishes like *Clarias gariepinus* (Angka et al., 1995), *Labeo rohita* (Sahu et al., 2007), *Sparus aurata* (Reyes-Becerril et al., 2011), *Magalobrama amblycephala* (Tarn et al., 2015). The severity of pathogenesis or disease outbreak is moderate to high in fishes (Yardimci and Aydin, 2011), but every type of disease results in deterioration of product and economic losses. A high mortality was reported among thermal stressed fishes due to *Aeromonas* infection (Ortega et al., 1995; Noga, 1996). At the same time, studies were also conducted to understand the various pathophysiological changes on *A. hydrophila* infection and overall immune function of fishes (Das et al., 2011; Reyes-Becerril et al., 2011; Zhang et al., 2015). To find a solution for these problems Knowledge about the defence mechanism in fishes against *A. hydrophila* can be very beneficial to control and prevent the disease outbreaks (Peatman and Liu, 2007a).

So far there are 6 recognized subspecies of *A. hydrophila* (www.bacterio.net/), and more than 5000 research articles have been published on this single species (Janda and Abbott, 2010). Despite of much progress in knowledge about the bacterium, many questions are still unanswered (Joseph and Carnahan, 2000) like the host requirement, course and causes of pathogenesis, temperature requirement of the bacteria and even the severity of the disease (Janda and Abbott, 2010). Mostly, the bacterium is considered as mesophilic but also has been isolated from diseased
rainbow trout that lives at temperature range of 5-20 °C and also reported as psychrophilic in nature (Myrick and Cech, 2004; Shahi et al., 2013). The cold temperature that slows down the metabolic activity of the fish and increases vulnerability to the diseases may also favour the growth of *A. hydrophila* (Janda and Abbott, 2010).

Aeromonad septicaemia is characterized by diverse pathological symptoms such as dermal ulceration, fin haemorrhages, fin rots, red sores, haemorrhages and necrosis of the visceral organs, *etc.* (Cipriano et al., 2001; Yardimci and Aydin, 2011). The acute form of the disease may result in fatal sepsis without any symptoms (Yardimci and Aydin, 2011) while chronic infections may show the symptoms like hemorrhagic septicaemia with ulceration, inflammation, and dermal lesions (Cipriano et al., 2001). Liver and kidney tissues are the primary targets of bacterial accumulation and sepsis (Yardimci and Aydin, 2011; Sun et al., 2014). The liver may become pale and show greyish to greenish coloration with foci while kidney may engorge and become friable (Yardimci and Aydin, 2011; Laith and Najiah, 2013). The above mentioned symptoms and severity of the Aeromonad septicaemia is species and environment specific, factors such as host-pathogen interaction, temperature requirement of the bacteria, course of pathogenesis, and immunity to pathogenesis may vary for fish species (Wu et al., 2007; Janda and Abbott, 2010).

At the molecular level, various genetic components are involved in the determination of degree of response to bacterial infections in fishes. So a transcriptional overview of *Tor putitora* is required to understand the immunological reaction. The knowledge about these factors is extremely important to increase the understanding of its immune response to bacterial infections and to develop control mechanisms and therapy strategies towards any bacterial infections. By keeping all the foresaid points in mind this thesis was aimed to examine the transcriptome profile of *T. putitora* after challenging with *A. hydrophila* and to characterize gene expression changes in test samples in comparison to control. Further, the genes which are significantly expressing in the test are to be validated using the quantitative real-time PCR (qPCR) technique.
OBJECTIVES

Study Objectives

The innate immune system is the first line of defence and, therefore, an important protection against pathogens. However, it is not strong enough to suppress pathogens like adaptive response due to its nonspecific nature. In spite of this, not many innate immune genes have been cloned in fish which can be validated in other species for evaluating immune response. There must be some specific genes which express during pathogenesis and confer immunity to the Golden Mahseer after bacterial infection. These genes can be tracked by comparing the transcriptome profiles of *Aeromonas hydrophila* infected Golden Mahseer (*Tor putitora*) with the control sample. Differentially expressed genes having a putative role in immune response can be identified using *in silico* analysis and further validated using quantitative real-time PCR (qRT-PCR) assay. The present study was therefore undertaken to answer few key questions and two major objectives —

**Key questions**

I. What are the various physiological changes occur in Golden Mahseer (*Tor putitora*) on *Aeromonas hydrophila* infection?

II. Which of the particular genes those up-regulate and down-regulate in pathogenesis?

III. What is the expression level of immune-related genes in pathogenesis?

**Objectives of the study**

I. Identification of immune responsive genes expressed in a bacterial infection in Golden Mahseer using transcriptome profiling analysis.

II. Characterization of few candidate genes by quantitative polymerase chain reaction (qRT-PCR).
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