Introduction

This thesis focuses on the isolation and characterization of Myxovirus (Mx) resistance protein of Mrigal, *Cirrhinus mrigala*. *C. mrigala* is a major carp endemic to the polyculture system of the Indo-Gangetic riverine plain. Mrigal is one of the most revered delicacies in south-east Asian countries but is also a host to a wide range of pathogens. Functionally Mx proteins are widely studied antiviral protein belonging to the family of dynamins. The protein is found in the entire vertebrate kingdom and even in the invertebrates and is highly conserved. This protein is triggered by interferon (IFN) to act against a wide range of viruses, including influenza A virus (FLUAV). Hence the interferon system, fish viruses, Mx protein; its role in different stages of development as well as its expression kinetics in different tissues after polyI:C induction are discussed.

*Cirrhinus mrigala* (Mrigal)

Mrigal (*Cirrhinus mrigala*) is a carp endemic to the Indo-Gangetic riverine system and is amongst the three Indian Major Carps widely cultivated in the South-east Asian countries including India. In Latin, the word Cirrus means curl fringe. This species along with *Labeo rohita* (Rohu) and *Catla catla* (Catla) as the major carps, common exotic carps like *Hypophthalmichthys molitrix* (silver carp), *Ctenopharyngodon idella* (grass carp) and *Cyprinus carpio* (common carp) and minor carps like are cultivated in polyculture systems in India. The carps belong to the Cyprinidae family and along with minnows consist of eight percent of the world’s fish. Cyprinids are the largest of the freshwater families, and the second largest vertebrate family (with Gobidae-the gobies-the largest). Cyprinids are native to North America, Eurasia and Africa; fossil evidence suggests that this family may have originated in Asia in the Eocene (Nelson, 2006).
Phylum Vertebrata  
Subphylum Craniata  
  Superclass Gnathostomata  
    Series Pisces  
    Class Teleostomi  
    Subclass Actinopterygii  
      Order Cypriniformes  
      Division Cyprini  
      Suborder Cyprinoidei  
    Family Cyprinidae  
      Subfamily Cyprininae  
  Genus Cirrhinus (Oken)  

Cuvier (1817)

**Distribution**

Mrigal has long been a part of polyculture system in India; however records of its culture are only available at early part of 20th century. The traditional culture of the species was limited to eastern part of India up to 1950. However, after the technology of artificial propagation of the species, the distribution has spread to all over India. In the polyculture system of South-East Asian countries like Nepal, Thailand, Bangladesh, Lao People's Democratic Republic, Myanmar and Pakistan and also Sri Lanka, Vietnam, China, Mauritius, Japan, Malaysia, Philippines and the former USSR, Mrigal is one of the most favored and preferred species.
Fig. 1.1: Geographical distribution of Mrigal in India (FAO, 1979)

Fig. 1.2: Main distribution of Mrigal worldwide (FAO statistics, 2006)
**Morphology and Life cycle**

Mrigal is a bottom dweller and survives well in all types of freshwater habitat. Its body is bilaterally symmetrical and streamlined with blunt snout often with pores, body with cycloid scales, head without scales, mouth broad, prominent upper lip with not so distinct lower lips; a pair of short rostral barbells; rows of pharyngeal teeth; dorsal fin near end of snout, dorsal fin not forked, head bigger than the pectoral fin and caudal fin deeply serrated and anal fin not extending up to caudal fin.

The commencement of south-west monsoon is the breeding season of Mrigal, in natural open waters Mrigal usually breeds in shallow pockets or in bundhs adjacent to the riverine system. Spawning is seen in the banks of water bodies with a depth of 50-100cm, over clay, or even rock substrate. Eggs of Mrigal are non adhesive and non floating types. Hatchlings are usually surface or subsurface dwellers while fry and fingerlings are bottom dwellers. It is an illiophage in its feeding habit and stenophagus, major part of its food is decayed and detritus, while phytoplankton and zooplankton are also consumed. Mrigal is eurythermal and can tolerate a minimum temperature upto 14ºC. Compared to other Indian major carps (Catla and Rohu) Mrigal is a slow grower. Mrigal is heterosexual and attains maturity after two years (Jhingran and Khan, 1979). Fecundity of Mrigal is high and it normally ranges from 100000-150,000 eggs/kg body weight.

**Aquaculture and Production**

Fisheries in India are a very flourishing and promising sector with high economic returns. In the past six decades 11-fold increase in fish production i.e. from 0.75 million in 1950-51 to 10.07 million tones during 2014-2015 has been observed. Globally India ranks second in the aquaculture production, and it has escalated from 46 percent in the 1980s to over 85 percent in recent years in total fish production (FAO 2015). About 70 to 75 % of bulk production total freshwater fish production is contributed by Indian Major Carps, namely Rohu (*Labeo rohita*), Catla (*Catla catla*), Mrigal (*Cirrhinus mrigala*), followed by common carp, Grass carp, Silver carp and Catfishes that contribute the balance 25 to 30% ( FAO 2015). The national average production levels
from stagnant freshwater bodies has increased from about 600 kg/ hectare/ year in 1974 to over 2900 kg/ hectare/ annum 2013 and there are reports of even escalated production of levels of 8-12 tonnes/ hectare/ year (Handbook of Fisheries and Aquaculture, 2013, ICAR publication, India). The technology of induced carp breeding and polyculture in static ponds has increased the production in freshwater aquaculture sector to an enormous extent.

Mrigal forms an important part of the polyculture system as well as sewage fed carp culture system. Being a bottom dweller, it is difficult to harvest all through normal harvesting customarily used for Carps. Still Mrigal has a good local market in India and abroad. Though, an increase in total global Mrigal production was reported till 1996, but it was relatively stagnant between 1996-2003. India is the leading producer of Mrigal followed by Bangladesh.

![Graph showing global Mrigal production](image)

Fig.1.3: Rapid expansion of Mrigal global production was stagnant after 1996 up to 2003 with highest of 552000 tonnes in 2000 and 445000 tonnes in 2001 (adapted from FAO, 2015)

India being the world leader in Mrigal production, huge revenues are being generated by this white carp in Aquaculture industry. An expected two fold increase in Mrigal production by 2015, has led to indiscriminate use of organic wastes, feed, antibiotics and chemicals. Carp culture ponds being closed systems, if there is disease outbreak both horizontal and vertical transmission of disease is possible. Due to intensive
Aquaculture disease can spread through water currents and fishing nets in nearby farms. Even more harmful is the vertical disease transmission when disease is transmitted from the diseased broodstock to the eggs, fry and fingerling which are transported to the uninfected areas. Hence a proper understanding of the fish immune system is very essential to know the pathway of disease transmission and its prevention.

Worldwide every year huge economic loss is incurred, due to viral diseases in carp family. Almost all virus families except Bunyaviridae, Flaviviridae, Paroviridae and Poxviridae infect teleost fish (Essbauer and Ahne, 2001).

**Table 1.1 Wide spectrums of viruses attacking different types of fishes and diseases caused by them**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Virus genome</th>
<th>Fish viral disease</th>
<th>Interferon and Mx</th>
<th>Susceptible host</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Betanodaviruses</td>
<td>positive stranded RNA viruses</td>
<td>Viral encephalopathy and retinopathy</td>
<td>Upregulation of Mx and IFN in brain of sea bass, Atlantic cod turbort</td>
<td>Sea bream, sea bass, Atlantic cod, Turbot</td>
<td>Scapigliati et al., Park et al., Rise et al</td>
</tr>
<tr>
<td>Isavirus</td>
<td>Negative sense single stranded RNA</td>
<td>Inflammation of liver and spleen, haemorrhaging and anaemia</td>
<td></td>
<td>Salmons</td>
<td>McBeath, 2007</td>
</tr>
</tbody>
</table>

Every year huge loss is incurred due to viral diseases in aquaculture. Some virus infect the young fish e.g. Infectious Pancreatic Necrosis Virus (IPNV) in salmonids, while others can attack at any point of the life cycle causing mass mortalities e.g. Viral Hemorrhagic Septicemia virus (VHSV) and Infectious Salmon Anemia virus (ISAV). Many fish viral pathogens do not show symptoms of the disease but remain in the host in a dormant state waiting for the right opportunity. These carrier fish can shed virus into the environment and be a constant storehouse of infection for other fish. However the exact mechanism of viral invasion and its host response in teleost is relatively a gray
area compared to its study in mammals. However viral diseases affecting Indian Major Carps has not been reported except the incidences of rhabdovirus infection in EUS affected fish. Frequent viral infection in Shrimp aquaculture is prevalent in Indian Sub-continent. As KHV and SVC are major concern in fishes, the present study was aimed to establish the antiviral mechanism of the mrigal by polyI:C model.

Lot of bacterial disease being encountered in the Carp polyculture system causing symptoms like red disease, haemorrhages, Septicemia etc. These symptoms are caused by *Aeromonas hydrophila*, *Edwardsiella tarda*, *Flavobacterium columnare* and *Staphylococcus aureus*. Bacterial disease is a major concern of intensive aquaculture system of Odisha, West Bengal and Andhra Pradesh

The innate defenses against virus can be either constitutive or responsive. A constitutive innate anti-viral response in fish comprises the action of non-specific cytotoxic cells to virus infected cells, and a responsive mechanism is interferon production (IFN) which is induced by virus infection (Ellis, 2001). Thus a comparative model of mammalian immune system will throw light into the teleost immune system and its response to virus.

**The Mammalian Immune System**

As compared to teleost immune system, the mammalian model is studied in detail; hence for better understanding of immune mechanism in fish a brief overview of mammalian immune system is very essential. The immune system of higher vertebrates comprises of primary and secondary lymphoid organs. The primary lymphoid organ comprises of bone marrow and thymus and the secondary lymphoid organ is mucosal associated lymphoid tissue (MALT). In the primary lymphoid organs, the lymphocytes are produced while in the secondary lymphoid organs lymphocytes are maintained and adaptive immune response is generated. In mammals, bone marrow is the haematopoietic tissue from which proliferates all types of red blood cells. Myeloid progenitor cells give rise to basophils, eosinophils, granulocytes, erythrocytes and platelets precursor megakaryocytes. While a lymphoid progenitor produces precursor cells for natural killer cells (NK cells) and lymphocyte precursors, various types of
dendritic cells also proliferate from these progenitor lines. Though B and T-lymphocytes are produced from the bone marrow, it is only T-lymphocyte that undergoes maturation in the thymus. Then the mature lymphocytes are carried by the blood stream to the peripheral lymphoid tissue. When a pathogen enters the body it is ingested by the dendritic cells and macrophages and then it is presented as antigens to the lymphocytes (Janeway et al., 2005). Myeloid and lymphoid immune cells coordinate to obstruct the pathogenic attack by activating a series of defence signaling pathways to preserve the tissue/organism structural and functional integrity (Candeias and Testtard, 2015). Myeloid cells such as neutrophils, dendritic cells, monocytes and macrophages are the primary soldiers of the innate immune system, that sense the presence of pathogen associated molecular patterns (PAMPs) by their pathogen recognition receptors (PRRs). However more recently the damage caused due to exposure to nanoparticles, UV radiations, heat exposure and protection of the organism against such injuries is possible due to release of the danger signals or Danger Associated Molecular Patterns (DAMPs) can also be recognized by PRRs (Candeias and Testtard, 2015). Activation of the complex pathways is essential to maintain diseases free conditions however persistent stimulation of these pathways have detrimental effect on the host like rheumatoid arthritis, cardiovascular disease and cancer (Gajewski et al., 2013)

Myelopoiesis in the teleost occurs in head kidney or spleen. Red bone marrow or lymph nodes as primary and secondary organs are absent in teleosts. The head kidney is the primary site for antibody production and also serves as a secondary lymphoid organ in the clearance of soluble and particulate antigens from the circulation (Whyte, 2007). Spleen also has a prominent role in antigen presentation and brings about the initiation of adaptive immune response (Whyte, 2007). Mammalian immune system unlike fish immune system is not completely dependent on innate responses. They have strong memory responses and have a wide range of antibodies. Though fishes have a repertoire of B and T cells their antibodies are not as diverse as in mammals. In contrast the innate immune system in fish is more evolved as it has several isoforms of C3 complement while in mammals there is only one. The growing interest in fish models for understanding human diseases is due to the homology between innate
organisms in fish and mammalian immune system. Further the primary immune molecules, Ag-presenting lymphocytes, immunoglobulins, MHC products, recombination activating genes and recombination pathways are homologous in fish and mammals (Rauta et al., 2012).

Table 1.2: Showing the comparison between immune system of Jawed fishes and mammals adapted (from Tort, Immunologia, 2003).

<table>
<thead>
<tr>
<th></th>
<th>Jawed fishes</th>
<th>Mammals</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Physico-chemical parameters</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature</td>
<td>-2-35°C</td>
<td>36.5 °C-37.5 °C</td>
</tr>
<tr>
<td>Habitat</td>
<td>water</td>
<td>Air</td>
</tr>
<tr>
<td>Metabolism</td>
<td>Poikilothermal</td>
<td>Homeothermal</td>
</tr>
<tr>
<td>External contact</td>
<td>Skin, gills</td>
<td>Respiratory tree</td>
</tr>
<tr>
<td><strong>Humoral diversity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ig isotypes</td>
<td>IgM, IgD (Teleostei)</td>
<td>IgM, IgA, IgD, IgE, IgG</td>
</tr>
<tr>
<td></td>
<td>IgM, IgX/ IgR, IgW, NAR (Chondrichythes)</td>
<td></td>
</tr>
<tr>
<td>Non-specific diversity</td>
<td>Several C3 isoforms</td>
<td>No C3 isoforms</td>
</tr>
<tr>
<td><strong>Immune response</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antibody affinity</td>
<td>low</td>
<td>High</td>
</tr>
<tr>
<td>Antibody response</td>
<td>slow</td>
<td>Fast</td>
</tr>
<tr>
<td>Memory response</td>
<td>Weak</td>
<td>Strong</td>
</tr>
<tr>
<td>Affinity response</td>
<td>Low or absent</td>
<td>High</td>
</tr>
<tr>
<td>Affinity maturation</td>
<td>dependant</td>
<td>independent</td>
</tr>
<tr>
<td>Temperature</td>
<td>temperature dependant</td>
<td>Involution with ages</td>
</tr>
<tr>
<td>Haematopoietic tissue</td>
<td>Head kidney</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>Thymus</td>
<td>Involution with species</td>
<td>Involution with ages</td>
</tr>
<tr>
<td></td>
<td>temperature dependant</td>
<td></td>
</tr>
<tr>
<td>Lymphoid nodes</td>
<td>Absent</td>
<td>Present</td>
</tr>
<tr>
<td>GALT</td>
<td>Not organized</td>
<td>Organized, Peyer patches</td>
</tr>
</tbody>
</table>
Mammalian Antiviral Immune Response

In mammals antiviral response is either through protein based IFN response or through RNA interference (RNAi) (Sagan and Sarnow, 2013). The existence of operational RNAi pathway is seen in plants and invertebrates and also in mammals suggesting that it is evolutionarily conserved. The inflammatory signals are recognized by germ-line encoded receptors including the Toll like receptors (TLRs), Nod –like receptors, Aim2 like receptors, Rig-I like receptors and C-type lectins present within distinct locations within the cell (Sasai and Yamato 2013). IFN system in mammals is able to combat almost all types of viruses hence is very profound and most studied. Identification of viral nucleic acids triggers the release of inflammatory cytokines, chemokines, and IFNs through the activation of NF-κB and IRF 3/7 pathways. The dynamin like GTPases genes Myxovirus 1 (Mx1) and Mx2 defend mammals against a wide spectrum of viruses. Failure of Mx1 function in human and mice increases the chances of infection by RNA and DNA viruses, including orthomyxoviruses (influenza A), paramyxoviruses (measles), and hepadnaviruses (hepatitis B) while mutation in Mx2 surges the chances of HIV-1 infestation (Braun et al., 2015). It has been reported by Langevin that IFN-stimulating signaling pathways are conserved in teleosts and mammals. Regarding the sensors, RLRs in fish are almost analogous to mammalian counterparts while except for TLR3 and TLR7 that are highly conserved in mammals, many fish TLRs s are absent in mammals.

The Teleost Immune System

Morphologically, the structure of immune system of teleosts is dissimilar to that of mammals. The most pronounced variance is that fish lack lymph nodes and bone marrow and head kidney is the primary lymphoid organ assisted by thymus, spleen and mucosa associated lymphoid tissue. The immune system of fish is also mediated by the changes in the aquatic environment as it is poikilothermic (Rauta et al., 2012). Migratory and non-migratory cell populations form the lymphoid tissues. The specific
and nonspecific immune responses are due to lymphocytes, monocytes, macrophages, granulocytes and thrombocytes (Ellis, 1977). Lymphocytes are further categorized as immunoglobulin-positive B cells and immunoglobulin-negative T cells (Miller, 1998) and other cells include mast cells, nonspecific cytotoxic cells and dendritic cells (Reite, 1998). The periportal tissues of liver and the haematopoietic tissues of spleen and kidney are rich in Melanomacrophages a distinctive population of pigment containing cells (Wolke, 1992). In teleost, the lymphatic system is different from that of blood vascular system (Wardle, 1971), and lymph collected from these vessels contained leucocytes, in same proportion as that in blood but erythrocytes was absent (Ellis & de Sousa, 1974). Mechanisms that are involved in providing short term immunity to viral hemorrhagic septicemia virus (VHSV) in rainbow trout after administration of DNA vaccine has been studied by Bela-ong (2015). MicroRNAs (miRNA) are short (18-22 nucleotides long) RNA that mediate gene expression by either inhibiting translation of or vitiating target mRNA resulting in a modified protein expression profile. Each miRNA regulates multiple genes including the development of immune cells and host parasite interface. It has been reported by that miR-462 and miR-731 support the IFN-mediated antiviral activities (Buggele and Horvath 2013; Bela-ong et al., 2015). Increasing business in Aquaculture, the coherent up regulation of diseases and also Zebra fish as model for human diseases as increased the importance of the study of fish immune system and its antiviral mechanism.

Thymus

Teleost thymus is a bi-lobed lymphoid organ, located in the dorso-lateral region of the gill chamber adjacent to the opercular cavity. The thymus is usually marked by outer cortex that has a high density of thymocytes and an inner medullar region that is rich in epithelial cells, mostly reticulocytes and epitheliocytes. However the two regions are not well demarcated in all teleosts hence it can be difficult to visualize. According to Chilmonczyk, 1992 epithelial cells release certain factors that are essential for thymocytes maturation and attainment of specific immune characters. The involution of thymus with age as seen in other vertebrates is not a common feature in fish; however involution of thymus is seen in spawning females and also varies with release of
hormones and seasonal changes (Nakanishi, 1986). The thymus is the first organ ontogenetically to become actively lymphoid although the timing is species dependent (Laird, 1978; Manning, 1994). In rainbow trout thymic primordium is seen just before hatching and at hatching lymphocytes can be seen in the growing thymus which is covered by an impermeable capsule (Heinecke et al., 2014). In salmonids the development of thymus, posterior and anterior kidney begins before the hatching of the larva. Thus it appears as an enlargement of the gill chamber above the three gill arches (Heinecke et al., 2014). The zebrafish thymus is active about three days post fertilization when lck and rag are present (Langenau et al., 2004). The thymus is also the first lymphoid organ to appear phylogenetically, occurring in cartilaginous fish (sharks and skates) after the proposed second round of WGD approximately 450 million years ago.

**Head Kidney**

As in all vertebrates, kidney is a paired organ located peritoneally, exterior to the dorsal body cavity consisting of a head kidney and trunk kidney. Head kidney is the lympho-myeloid agglomerular part devoid of excretory activity and trunk kidney is the glomerular compartment. Head kidney is the haematopoietic organ having functionally analogous to the bone marrow of higher vertebrates (Meseguer et al., 1995). Head kidney consists of large number of T-lymphocytes, B-lymphocytes, granulocytes and macrophages. Head kidney is the secondary lymphoid organ - a lymph node analogue, essential for the stimulation and upregulation of immune responses (Kaattari & Irwin, 1985). B cells derive from the head kidney or pronephros in teleosts which is the anterior part of a long organ responsible for many functions, including that of a mammalian kidney. The stroma of the head kidney is an important haematopoietic tissue, has a vital role in non-specific immunity and facilitates in removal of cell debris and damaged cells (Whyte, 2007). Macrophages and B cells responsible for antigen recognition and presentation are confined in the anterior kidney of teleosts (Rauta et al., 2012). After injection of radio labeled bacteria into rainbow trout, about 70% radioactivity is noticed in the head kidney (Ferguson et al., 1982). The sinusoidal macrophages and the endothelial cells work as filter beds, for the sieving of blood borne
particle (Brattgjerd & Evensen, 1996). Antibody production takes place in the head kidney, and the presentation of antigens is done by melanomacrophage of the parenchyma (Brattgjerd & Evensen, 1996), which might play an eminent role in immunological memory (Press et al., 1996). The kidney is a vital tissue for processing of pathogens in teleosts and many researches are going on to understands the kidney functions and molecular gene expression. A study was carried out by Chetri (2011) to understand the differential response of head kidney leukocytes from rainbow trout to different PAMPs to poly I:C, bacterial (flagellin and LPS) and fungal infections (zymosan and β-glucan) and it was found that there was a specific signaling pathway activating different immune effector molecules. Head kidney of salmon may act as a secondary lymphoid organ where antigen presentation takes place (Iliev et al., 2013). A significant up-regulation of TLR5 in the kidney at 28dp.v. of live attenuated Vibrio anguillarum MVAV6203 vaccine increased the immunity of Zebrafish(liu et al.,2015) . It is studied by (Tong et al.,2015) that kidney play a vital role in maintaining the osmotic, electrolyte and water balance and transcriptome analysis of naked carp, Gymnocypris przewalskii found a set of sodium calcium expressing gene.

**Spleen**

The spleen is a secondary lymphoid organ having function similar to the head kidney, with respect to antigen trapping and removal of blood borne substances. In teleosts, spleen has a prominent red pulp and white pulp. The red pulp is rich in macrophages and lymphocytes and white pulp is comparatively under developed and mostly has an accretion of melanomacrophage and ellipsoids (Secombes & Manning, 1980). Similar to the germinal centres of higher vertebrates, melanomacrophage retain antigen complex for extended periods in the form of immune complexes (Ferguson, 1976; Agius, 1980). While melanomacrophages are the sites for erythocyte destruction (Zapata & Cooper, 1990), ellipsoids have special function in plasma filtration and retention of immune complexes (Espenes et al., 1995b). Proliferation of granular cells has also been observed after immunisation in association with ellipsoids and melanomacrophage centres (Van Muiswinkel et al., 1991). Interestingly, clustering of RAG expression has been identified in the spleen of rainbow trout using in situ hybridization, suggestive of
germinal centre formation but this has not been confirmed (Hansen and Zapata, 1998). In teleosts immune cells like macrophages and B cells responsible for antigen presentation are mostly confined to the anterior kidney and spleen (Rauta et al., 2012). Sun (2011) reported high upregulation of interleukin 1β, natural killer cell enhancing factor, Mx, MHC1α and IgM in spleen of Japanese flounder after vaccination with *Edwardsiella tarda* antigen. It has been reported that the expression of TLR5 significantly increased in kidney and spleen of common carp after *A. hydrophila* infection (Duan et al., 2013). The spleen of teleosts like the mammals plays a vital role in the protection against blood borne pathogens. The T and B cells block the further manifestation of the disease in the host cell by neutralizing the antigen, thus spleen is an important organ to study the immune parameters. Thus transcriptome profiling of several aquaculture species (orange spotted grouper, large yellow croaker, turbot, Japanese sea bass and rohu carp following exposure to pathogens have been studied to understand the functioning of immune genes (Li et al., 2015).

**Mucosal Associated Lymphoid tissue (MAL T)**

The mucosa associated lymphoid tissue includes the gills, gut and skin which form the first line of defence for the invading pathogens. The mucus is rich in antimicrobial peptides (AMP) and enzymes rich in innate immune response. The innate immune response is the primary response until specific antibodies are triggered like the cytotoxic T-cells and antibodies.

**Gill**

The primary respiratory organ in the teleosts is gills. They are in the continuous contact with the aquatic environment that is a storehouse of pathogens. MHC class II expressing cells have been identified in gills and their numbers increase during amoebic gill disease indicating that the gills are active immunologically (Koppang et al., 2003; Morrison et al., 2006). Recently, a lymphoid tissue at the base of the gill arch has been reported which exhibits localised CD3, TCR and MHC class II expression (Haugarvoll, 2008). Fish gills are mesh like structures, and functions in trafficking of ions, electrolytes and also the exchange of oxygen, carbon dioxide and ammonia. Tong (2015) in the
transcriptome analysis of *Gymnocypris przewalskii* identified certain gill specific genes like the cation transporter, urea transporter and calcium ion transporter.

**Hind Gut**

Teleost intestinal mucus has some functions similar to the gut, gills and skin apart from acting as a sieve and preventing the entry of particulate matter including microorganisms. Nutrient uptake and digestion is mediated by gut mucus (Bakke et al., 2010). In air breathing fish oxygen absorption from air is done by gut mucus (Nelson and Dehn, 2010). Innate and adaptive immune factors such as antimicrobial molecules or secretory immunoglobulins are present in gut mucosa (Salinas et al., 2011 and Gomez et al., 2013). Peyer’s patches and mesenteric lymphatic nodes unlike mammals are absent in teleosts but the gut is rich in populations of leucocytes, including macrophages, lymphocytes, mast cells, granulocytes and plasma cells (Georgopoulou & Vernier, 1986). Maximum leucocyte concentration is seen in the lamina propria of the gut folds and epithelial leucocytes are found in the gut (McMillan & Secombes, 1997). In most of the teleost species a row of mast cells/ granule cells is sandwiched on one side by the stratum compactum and the other side by the circular muscle layer (Reite, 1997). T cells expressing TCRβ, TCRγ, CD3, CD4 and CD8, but not IgM have been isolated from teleost mucosa (Bernard et al., 2006). Gut epithelial cells are protected by mucus layer that play a vital role in innate defence and maintains tissue homeostasis (Gomez et al., 2013). The secretion of mucin by goblet cells in the intestine is a continuous process that leads to the removal of attached pathogens or toxins (Jutfelt, 2011).

**Skin**

The teleost skin is the organ that not only protects it in the aquatic environment from the invasion of pathogens but also works as a metabolically active tissue covering the entire body of fish including fins. Being an important organ in the first line of defense, it plays an important role in perceiving sensory impulses, locomotory activities, respiration, ion-uptake, excretion and temperature monitoring (reviewed by Eliott 2000 and Marshall, 2010). The impertinant nature of skin mucus prevents the entry of
bacteria, viruses and other pathogens into the skin of the fish. The inherent property of epidermal mucus to inhibit infectious pathogens (bacteria and viruses) has been demonstrated in different fish species (Su, 2011 and Wei, 2010), and upregulated expression of antimicrobial components in fish epidermal mucus has been reported following microbial stress (Patrzykat, 2001). One of the most studied skin responses is seen in case of lymphocystis that is characterized by light colored nodular lesions on the skin. Some symptoms of viral diseases in fishes; European catfish herpes virus a thickened epidermis without mucous cells; Carp pox disease is characterized by focal lesions benign nonnecrotizing epidermal hyperplasia (Coffee et al., 2013). Fish alloherpesvirus is characterized by epidermal cell necrosis, papillomas and syncitia formation (Hanson et al., 2011).

**The ontogeny of the immune system of teleosts**

With the increase interaction between the host and the pathogen, the vertebrate immune system has evolved in terms of antigen receptors and MHC molecules. With the evolution of jaw in vertebrates, the development of immunocompetent cells and its role in defence mechanism in primary and secondary lymphoid organ changes. The immunocompetent cell develops from a common hemangioblast that can produce hematopoietic stem cells (HSC) and endothelial cell progenitors. These HSC later give rise to different type of blood cells including B- and T- lymphocytes. In order to understand the level immunocompetence of fish at different stages of its life cycle it is very essential to study the ontogeny of the immune system.

**Intermediate Cell Mass**

In all vertebrates, blood morphogenic protein signalling stimulates the ventral mesoderm in to synthesize blood. These hematopoietic tissues give rise to different haematopoietic cells and the adult lymphoid cells. In the developing embryo blood cells appear in the ventral region of tail two days post fertilization. The blood cells are surrounded by the primitive macrophages and / or myeloid cell in the connective tissue. The
hematopoietic activities of these embryonic regions have been studied in the zebrafish by whole mount in situ hybridisation (Trede, 2001).

**Immune response of teleosts**

It is an accepted fact that the teleost immune system possesses the components of both adaptive and innate immunity as seen in higher vertebrates like mammals. Traditionally the complex immune system can be divided into the ancient innate immune system, found in all multicellular organisms and comparatively recently developed adaptive immune system that arose with the appearance of jawed vertebrates Chondrichythes (Kasahara, 2004).

**Maternal Immunity**

Maternal immunity refers to the immunity transferred to the offspring across the placenta or through milk, yolksac, colostrum to the neonates which is very essential for the survival of the immune-imcompetent young ones. Teleostan embryos during the early developmental stage are dependant on the adult female for the arrangement of zona pellucida that acts as a barrier for defence. Secondly it also mediates the supply of nutrients, maternal hormones and antibodies that are known to play a vital role in early immune competence of the embryo (Li and Leatherland, 2012). While most transcripts may be transferred to the oocyte, the oocyte transcriptome is dynamic and is totally controlled by the accumulating and degrading mRNA (Sanchez and Smith, 2012). Equally important is the masking of the transcripts so that they are not expressed prematurely (Conti, 2011). Thus with the beginning of embryogenesis maternally inherited factors like the mRNA and micro RNA are control the translation as well as the selective degradation of the maternal RNA (Giraldez et al.,2010). Fish age is a determining factor for mortality of larvae, as many viruses are known to attack at this early stage when the immune system is not fully developed. This is particularly observed in case of many viral diseases occurring in fish larvae like the infectious pancreatic necrosis virus (IPNV), infectious hematopoietic necrosis virus (IHNV) in salmonids, and viral nervous necrosis virus (VNNV) (Matsuyama et al.,2012). Mass mortalities are common feature for hatchery reared larvae and juvenile as due to the poorly understood fish immune system and absence of vaccines to be administered to larvae or
young fish. Transfer of maternal immunity has been studied in some fishes. In brown trout, mRNA transcripts of Lysozyme were observed in unfertilized egg (Cecchini et al., 2013). Lysozyme activity protects the oocyte and eggs against vertically transmitted bacterial diseases. Maternal transfer of IgM and other complement factors have been studied in rainbow trout and cells positive for membrane IgM have been seen in kidney 4-8 days post hatching (Heinecke et al., 2014).

**Antiviral Immune Response in Teleost**

The innate immune system is the only pivotal immune defence system of invertebrates and major defence system of fish. The innate immune system is comparatively older, found in all multicellular organisms and it has an instructive role for adaptive immune system and homeostasis. A study on the fish and mammalian immunology highlights that innate and adaptive immune system work in close association for a wide range of immune activities (Flajnik, 2004). Evolutionarily Carps and other teleost are long way ahead of mammals and hence very little is known about the immune system and immune responses. Teleosts evolution is the time when classical adaptive evolution was just discovered. Thus the teleosts form an interesting model in understanding the immune responses. Thus fundamentally the primary role of the innate immune system is to act as first line of defence barrier against pathogens and simultaneously modulate certain factors to instruct to the adaptive immune response to take over the defence mechanism (Vasta 2002). Fishes being poikilothermic there is a constraint of antibody repertoires, affinity maturation and comparatively slow lymphocyte proliferation (Magnadottir, 2006). The pathogen recognition receptors (PRR) of the host cells recognizes and binds to the conserved pathogen associated molecular patterns (PAMPs) such as peptidoglycans, lipopolysaccharides (LPS), C-reactive protein, mannann-binding lectin, mannose receptor and β-1,3-glucans, which are absent in eukaryotic cells but common to all pathogenic microorganisms (Ellis, 2001). Once the PAMPs has been recognized by PRRs, Toll-like receptors several intracellular signaling pathways are triggered that results in the up regulation of genes involved in antiviral responses, inflammation and maturation of dendritic cells (Whyte, 2007). Studies of host response to viral infection in bony fish and mammals are almost identical in the mechanism
involving both innate and adaptive immune responses against viruses. In the struggle between a host and a virus, the host stimulates orchestras of immune response and the virus tries to activate its virulence factors that will help it to evade the immune mechanism of the host. Thus several viruses are active by antagonizing both innate and adaptive immune response. Thus the ability of viruses to manifest a disease depends upon its capacity to replicate in the host cell using host cellular mechanism and evading potent host immune response.

Viral diseases cause significant losses in aquaculture. Some viruses mainly affect young fish like IPNV (Infectious Pancreatic Necrosis Virus) in Salmonids while other viruses affect the fish all throughout the life eg. Viral Haemorrhagic Septicemia (VHSV) and Infectious Salmon Anaemia Virus (ISAV). Almost all virus families except Bunyaviridae, Flaviviridae, Paroviridae infect teleost fish (Essbauer & Ahne, 2001). Depending upon the type of host and its immune system, the severity of viral disease and its effect varies. The innate immune response detects the Viral Associated Molecular Patterns (VAMPs) in association with the germ-line encoded pattern recognition receptors (PRRs). The functionality of adaptive immune response depends upon its wide spectrum of receptors generated by somatic gene rearrangement to identify the pathogens and give the host an immunological memory (Janeway and Metchnikov, 2002). A virion is an agglomeration of nucleic acids (DNA or RNA) and protein while enveloped viruses also have lipids. The protein are mostly structural proteins like capsid proteins, matrix proteins, membrane glycoprotein’s and other accessory proteins like kinases, RNA polymerases, reverse transcriptases and proteases. The capsular protein is either helical or icosahedral and protects the RNA or DNA genome. In case of enveloped viruses, the lipid bilayer membrane protects the, capsid, the nuclear material and also behaves as a transport vehicle during cellular transmission. Viruses being the connecting link between living and non-living are require host cellular machinery for replication. Thus viruses need to transit the plasma membrane barrier of the host, for which they have to bind to the cellular receptors. The membrane fusion of encapsulated virus with the host plasma membrane and the nucleic acid is carried into the host cell. In case of non-enveloped virus either through membrane lysis or pore
formation in viral nucleic acid enters the host cell. Inside the cell, after transcription/ or replication process of viral nucleic acids such as dsRNA and ssRNA, expression of viral proteins will take place. Cellular mRNAs are eukaryotic mRNA which are capped structures and are much shorter in length compared to viral RNAs which have a 5’phosphate moiety and are greater than 30kb so that they can be recognized by cellular PRRs. Unmethylated bacterial DNA with CpG motif (Klinman et al., 2004) and double stranded DNA with left handed Z-conformation mediated by RNA polymerases (Rich and Zhang, 2003) are also recognized by cellular PRRs. Thus ssRNA, dsRNA and viral glycoproteins constitute the basic VAMPs that are recognized by PRRs. According to Thompson and Locarnini (2007) multiplication of viral nucleoproteins and its presentation as VAMPs can occur at any point of viral cycle. This interaction of VAMP-PRR mediates the expression of a variety of cytokines including inflammatory cytokines, type I IFN, chemokines and to support the proliferation of T-cells.

Type I interferon play a pivotal role in the stimulation of antiviral response, which induces the expression of antiviral genes that ultimately leads to the translation of many IFN inducible genes. In fish, though IFN were identified many years back, but the first IFN sequences were only reported in the early 2000s (Altmann et al., 2003; Lutfalla et al., 2003; Robertsen et al., 2003).

**Interferons (IFN)**

The innate immune system is primary defence system in fish combating viral infections. A wide range of cytokines and chemokines are produced by several sort of host cells in response to viral infection, type I IFN being the principal cytokine. Isaac and Lindenmann (1957) reported that chick cells infected with influenza virus secreted certain chemicals that were capable in sensitizing other host cells against heterologous and homologous viruses. IFN like activity in fish was discovered way back in 1965 and since then it has been discovered in several fish species and cell lines after viral infection or induction by double stranded RNA (Robertsen, 2006). The advances in the research on the teleost IFN system has helped us in understanding the diverse areas of fish cell biology and biochemistry, ranging from pathways of signal transduction to transcriptional and translational control to viral disease pathogenesis and its probable
treatment. Teleost fish possess multiple type I IFNs to combat varied types of viruses. As in mammals, they are stimulated by viral attacks and antiviral responses are activated through numerous cell receptors. The IFN genes have been sequenced in many fishes like Salmon, Carp, Catfish, Puffer fish, Gold fish, Sea bass, Rainbow trout and Zebrafish (Zou et al., 2014).

Classification of IFNs

Most Based on their structural, functional and amino acid sequence the IFN are grouped into two families called TypeI and TypeII IFNs. To, date three types of IFN have been studied in vertebrates with type I and type III signaling through different membrane bound receptors but functioning through the main JAK/STAT pathway to trigger the response of a spectrum of antiviral genes (Randall and Goodbourn, 2008 and Robertsen, 2008). The mammalian immune system consists of type I, type II and type III IFN. Type I comprises of five subclasses; IFN-α, IFN-β, IFN-ω, IFN-ε, & IFN-κ; type II IFNs of IFN γ alone, and type III IFN of IFN-λ1, IFN-λ2 and IFN-λ3. Based on cysteine patterns, fish typeI IFN are further divided into two major groups, mainly group I and groupII. Interestingly the two groups of IFN signal through different receptors in Zebrafish. IFNφs of the first group (φ1 and φ4) bind to cytokine receptor family CRB1-CRB5 would protect against both bacteria and virus while groupII IFN (φ3 and φ4) are most effective against bacteria (Langevin et al., 2013). Type I IFNs include multiple IFN isoforms(13 have been found in man), a single to three IFN β. IFN-like cytokines limitin found only in mice, and interleukin-28A, IL-28B and IL-29 predominantly found in humans and other species (Pestka et al., 2004). IFN-α, IFN-β, IFN-ω, IFN-ε and IFN-κ are specific to humans while IFN-τ and IFN-δ are prevalent in pigs and cattle (Platinas, 2005). Type I IFN can be distinguished from TypeII IFN by their stability at PH2. Though structurally both types of IFN belong to the class II α-helical cytokine family, but their structures are different and they bind to different receptors. Fish IFNs are thought to be homologs of mammalian type I or III IFNs because they share the similar genomic organization with type III IFN genes, in which four phase introns are present (Zou et al., 2014). Due to the retrotransposition event in the evolutionary cycle of fishes, it can be speculated that there was a distinct
divergence in the typeI and typeIII IFN (Zou et al., 2007). However fish IFNγ are not always induced after viral attack and very less reports of their antiviral activity so only typeI IFN family will be discussed as potent inhibitors of virus.

**Type I Interferon family**

In 2003 first fish IFN was cloned by three different groups in Zebrafish *Danio rerio*, (Altmann et al., 2003), Atlantic salmon , *Salmo salar*, (Robertsen et al., 2003) and pufferfish (Lutfalla et al., 2003). Like mammals fish also possess multiple copy number of the gene. One of the distinguishing feature of fish type I IFN gene is its genomic organization, comprising of five exons and four introns which is strikingly different from reptiles, birds and mammals that have only single exon (Sun et al., 2009). The fish IFN are similar to mammalian IFN with 152- 164 amino acids corresponding to 18-19kDa. The IFNα/β receptors contain subunits IFNAR-1 and IFNAR-2 that belongs to ClassII receptor cytokine (CRFB) family.

**Bioactivity of fish IFN**

When virus enters a cell its basic genetic material DNA or RNA is exposed, which uses host machinery to multiply and then assembles into the viral capsid and leaves host cell to infect new cell. At this point the viral nucleic acids, derepresses the IFN genes, thus stimulating IFN production. An alternative theory was that cells contain precursors of IFNs that are stimulated by virus particles and IFNs are released into the extracellular environment where it binds to the cognate receptors on the neighbouring cells. This process of release of IFNs is much faster than the release of viral particles so that they can sensitize the neighbouring cells. Numerous signals are released to induce a wide range of target genes or IFN-stimulated genes, to elicit an antiviral response. The mechanisms of transcriptional activation of the IFN inducible gene have been well documented. IFNs have multi-faceted functions. They monitor the stimulation of intrinsic proteins like TRIM5α, Fv, Mx, elf2α and 2’-5’OAS, and that obstruct viral replication by either blocking viral transcription or bring about cellular apoptosis (Samuel, 2001). Concomitantly they are known to stimulate the expression of the adaptive immune system by activating NK cells and dendritic cells (Le bon, 2002). Type
I IFN induce the expression of a diverse types of IFN stimulated genes (ISG), Mx Viperin, PKR, ISG15 thus creating an antiviral state. Few studies have been done with recombinant fish IFNs that mimic the characteristics of type I IFNs. Transcripts of recombinant Salmon and Catfish IFN showed antiviral activity against infectious pancreatic necrosis virus (IPNV) and Channel Catfish Virus (CCV), respectively (Robertsen et al., 2003; Long et al., 2004). Zou have shown that recombinant trout, TypeI IFN is able to induce Mx gene expression in RTG-2 cells. Pufferfish and Salmon IFNs have shown to induce Mx protein (Robertsen, 2003; Lutfalla et al., 2003). Further in Zebrafish cells transfected with Zebrafish IFN an upregulation of Mx transcript and also resistance against infection to snakehead rhabdovirus was observed (Altmann et al., 2003).

**Induction of IFN by dsRNA and virus infection**

At cellular level the IFN system works in two modes; induction and action. When virus enters a cell its basic genetic material DNA or RNA is exposed, which uses host machinery to multiply and then assembles into the viral capsid and leaves host cell to infect new cell. At this point the viral nucleic acids, stimulates the IFN genes, thus riggering IFN production. An alternative theory was that cells contain precursors of IFNs that are stimulated by virus particles and IFNs are released into the extracellular environment where it binds to the cognate receptors on the neighboring cells. This process of release of IFNs is much faster than the release of viral particles so that they can sensitize the neighbouring cells and create an antiviral state. Viral dsRNA and polyI:C is identified by TLR3, ssRNA is recognized by TLR7 and TLR8 and TLR9 binds to viral and bacterial unmethylated CpG DNA motifs (Robertsen, 2006). In mammalian cell during virus attack, transcription of IFNβ is induced first through activation of transcription factor interferon regulatory factor (IRF) 3. Among teleost fish IRF-7 has been cloned in crucian carp (Zhang et al., 2003), studies on IRF-3 is going on. Moreover poly I:C is a potent inducer of Mx transcript in different species of fish tissues and also their cell lines(Robertsen, 2006). Jorgensen have reported that CpG DNA induce Mx protein and antiviral state in Salmon. Apart from viral glycoproteins and double stranded RNA, expression of IFNα/β is also induced by bacterial infection,
and by components of bacteria such as LPS and bacterial DNA having CpG motifs common to bacteria but not mammals. Poly I:C injection in VHSV infected rainbow trout slowed down disease development and decreased mortality by 60% (Bela-ong et al., 2015)

**IFN receptors**

The PRRs (Pathogen Recognition Receptors) including the TLRs, RLRs (Retinoic acid-inducible gene I like receptors) and NLRs (Nucleotide-binding oligomerization domain like receptors) discriminates between self and non-self nucleic acids after viral infection. Viral nucleic acids, including single stranded RNA (ssRNA), double stranded RNA (dsRNA) and DNA are recognized by TLR7, TLR8, TLR3 and TLR9 (Gerlier and Lyse, 2011). A novel member in TLR family TLR22 is analogous to mammalian TLR3 and recognizes double stranded RNA viruses (Zhu et al., 2013). Endosomal TLRs, TLR3, TLR7, TLR8 and TLR9 are activated by nucleic acid and their expression is triggered in the presence of IFNs. TLR7 recognizes a wide spectrum of RNA virus including orthomyxovirus in plasmacytoid dendritic cells. TLR3 recognizes ssRNA and acts through IFN β receptor. IFN receptors are members of the family of Class II helical cytokine receptors, with ligands IL-10 related cytokines and tissue factor. IFNAR-1 and IFNAR-2 are the subunits of IFNα/β and IFNγ receptor consist of two subunits IFGR-1 and IFNGR-2 (Robertsen, 2006). A study on fish IFN receptors in pufferfish has identified 11Class II HCR genes. It has been studied that IFNs are potent stimulators of antiviral response elicited by the identification of pathogen associated molecular patterns by host cells. However, recently IFNs have induced the expression of cellular miRNA in human cell.
Fig. 1.4: Mode of action of IFN, the activation of different signaling pathway and the inhibition of viral replication

**IFN signalling pathway**

Type I IFN can be synthesized by all nucleated cells in response to viral infection while type II IFNs is produced by T cells and NK (natural killer) cells. Type III IFNs, comprised of IFN-κ-1, κ-2 and κ-3, Type I and Type III IFN are directly induced by viral infection and stimulate the transcription of antiviral genes through the Jak kinases and STAT pathway. Immediately after viral infection, cellular production of IFNα/β is triggered either by double stranded RNA which is at least produced at some point during their replication cycle (Jacobs, 1996) or viral glycoproteins (Ankel et al., 1998). Type I IFN can be produced by any cell type like fibroblasts, leukocytes and endothelial cells. Although virtually any cell can produce IFNα/β after viral infection but there are some cells which express nearly thousand times higher IFNα/β after exposure to virus, these cells are called “Natural Interferon Producing Cells”. In addition to producing IFN in response to viruses, they also do so on exposure to CpG DNA (Krug et al., 2001). IFN mediated signalling and cellular transcription could be best studied through the JAK-STAT signalling pathway proteins. The Signal Transducer and Activator of Transcription (STAT) family of proteins are silent cytoplasmic factors that
get activated after tyrosine phosphorylation of the Janus family of tyrosine Kinase (JAK) after cytokine stimulation. Of the known JAKs and STATs, the Jak1, Jak-2 and Tyk-2 kinase and the Stat1 and Stat2 transcriptional factors play an eminent role in mediating IFN depended biological process including stimulation of anti-viral state (Samuel, 2001).

In host cells, viral nucleic acid or its intermediates are either detected by the RNA helicases or TLRs thus inducing the expression of Type I IFN genes. Type I IFNs have two receptors IFNARI and IFNAR2 associated with tyrosine kinase 2 (TYK2) and Janus Kinase 1 (JAK1) (Samuel, 2001). As Type I IFN binds to the IFNAR1, transphosphorylation and activation of TYK2 and JAK1 takes place. TYK2 phosphorylates IFNAR1 activating the docking site for STAT2. TYK2 then phosphorylates STAT2, followed by STAT1. The phosphorylated heterodimers dissociate from the receptors, enter the nucleus to begin the transcription of IFN stimulated genes (Workenhe et al., 2010). Very little work has been done on the IFN-signaling system of fish, JAK1 and TYK2 have been cloned from the puffer fish and STAT 1 has been cloned from zebrafish and crucian carp. Zebrafish STAT1 was able to presume type I IFN-signaling functions in a STAT1 deficient human cell line, likely to be conserved between fish and mammals (Verhelst et al., 2013). The signaling pathway that stimulates the induction of TypeI IFN depends upon the stimulus and the receptor cell types. The entire process releases some common signaling molecules like TNF receptor- associated factor 3 (TRAF3) and the transcription factors IFN regulatory factor (IRF3 and IRF7). IRF3 and IRF7 enter the nucleus and concurrently with the nuclear factor NF-κB bind to the promoters of IFNA and IFNB to initiate the transcription of IFN genes.

**Induction of TypeI IFNs by bacteria**

TypeI IFNs are secreted cytokines that are known to mediate a diverse range of immune response to different pathogenic infections. Although TypeI IFNs are known to be active against viral pathogens but they are also effective against a range of bacterial pathogens. Bacterial induction of TypeI IFNs is by two pathways

(i) TLR recognition of bacterial molecules like Lipopolysaccharide LPS-A

(ii) TLR independent recognition of different molecules that entered into the cytoplasm.
TLR4 recognizes lipidA moiety of Lipopolysaccharide of the Gram negative bacteria (Monroe et al., 2010). TLR4 induces TypeI IFN signalling in many cell types and TLR3 is also known to induce TypeI IFN signalling after polyI:C or double stranded RNA signalling. Gram positive bacterial cell wall can also stimulate innate immunity. TLR2 plays a vital role in recognition of bacterial components like peptidoglycan, Lipoteichoic acid that is present in both Gram positive and Gram negative bacteria. The CpG motif of bacterial genomic DNA are also an immunostimulant and is recognized by TLR9 (Akira et al., 2006). Thus TypeI IFN production is seen after stimulation of TLR3, TLR4, TLR7 and TLR9 ligands.

Several cytosolic pathways in which host cell cytosolic sensors induce TypeI IFNs that is stimulated by RNA, DNA or cyclic –di-GMP. Nucleotide binding and Oligomerization Domain (NOD) sense a wide range of bacteria or their products and play a vital role in innate immune response. In response to bacteria NOD signalling induces TypeI IFN production in union with NF-κB and other transcriptional factors. Thus TypeI IFN is induced by either secretion of certain molecules by viable bacteria or degraded or lysed bacteria in the phagosome.

Thus it can be said that the production of TypeI IFNs is an immediate innate immune response to bacterial infections and studies using pure IFNs have shown the antibacterial nature of IFNs.

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**Interferons induced proteins and their anti-viral mechanisms**

Type I and type III IFNs are produced after viral attack and stimulate the release of several ISGs which in turn trigger several antiviral pathways. Among these antiviral pathways are PKR, Mx GTPase and 2’5’ OAS are most studied.

i) **Protein Kinase PKR**

ii) **2’-5’Oligoadenylate Synthetase and RNase L**

iii) **RNA-Specific Adenosine Deaminase ADAR1**

iv) **Protein Mx GTPase**

**Protein Kinase Receptor PKR**

PKR is an IFN inducible, RNA dependant protein kinase predominantly found in the cytoplasm. PKR is activated by autophosphorylation and also catalyzes the phosphorylation of five different substrates; the subunit of protein synthesis initiation factor 2, eIF-2α (Samuel, 2001); the transcription factor inhibitor IκB (Kumar, 1994); the Tat protein encoded by human immunodeficiency virus (HIV) (Mc Millan, 1995); the 90-kDa NFAT protein (Langland, 1999); and the M-phase specific dsRNA-binding phosphoprotein MPP4 (Patel, 1999). Several studies have shown that phosphorylation of proteins is monitored by PKR known to play a pivotal role in antiviral action of IFN and also in cell growth. PKR are expressed in normal cells but are in dormant state and
are up regulated by type I and type III IFNs (Sadler and Williams, 2008). Viral PKR inhibits the regular cellular machinery of the infected cells thus catalyzing the apoptosis of infected cells (Zhou et al., 2013).

**2′-5′ Oligoadenylate Synthase and RNase L**

OAS1 is activated by dsRNA which in turn activates the ribonuclease L (RnaseL) that degrades both cellular and viral RNA (Haller et al., 2007). The IFN-inducible 2-5 Oligoadenylate, degrades viral RNA with the help of two enzymes, OAS and RNaseL. Oligoadenylate possess 2′-5′ phosphodiester bond linkage, RNaseL enzyme is activated by binding to 2′-5′ oligonucleotides. RNaseL is constitutively present in all cell types and is known to inhibit the family of Picornavidae. It obstructs the basal cellular processes thus mediating cell death of virally infected cells (Zhou et al., 2013).

**RNA specific Adenosine Deaminase ADARI**

The viral RNA and cellular RNA regular activities can be changed by post transcriptional RNA modification such as deamination of adenosine to yield inosine. RNA A-to-I editing produces RNA transcripts in which I is recognized as G and not A by polymerases and ribosomes. This deamination of RNA modifies the regular protein codes of amino acid thus altering the transcript and the sequence of replicated RNAs (Zhu et al., 2013).

**Mx Protein and GTPases**

Mx1 and MxA proteins are members of the dynamin superfamily, and are most widely studied anti-viral proteins that have the potential to block the synthesis of viral nucleo capsids. Mx proteins are GTPases that have a molecular weight of 70-80kDa, and the GTPase activity is intrinsic for its antiviral mechanism. The human MxA inhibits a spectrum of RNA viruses. It accumulates in the cytoplasm of IFN treated cells and together with endoplasmic reticulum inhibits the transfer of nucleo capsid of bunyavirus to the Golgi apparatus where the virus assembly takes place (Zhu et al., 2013). There are a band of IFN induced proteins in virus infected cell, like PKR, 2′-5′ Oligoadenylate Synthase (OAS) and RNase L, RNA-specific adenosine deaminase...
(ADAR) and the Mx protein and GTPase. Protein phosphorylation, RNA degradation is mediated by dsRNA, RNA editing is catalyzed by the IFN inducible enzymes the PKR kinase, the OAS synthetases, and the ADAR1 deaminase also inhibit viral replication (Samuel, 1998). Up regulation of Mx expression in cells is either an indication of IFN synthesis or antiviral response to viral attack (Thanasaksiri et al., 2014). Induction of Mx gene expression \textit{in vitro} and \textit{in vivo} after poly I:C stimulation is very well documented (Peng et al., 2013). In case of teleost, the role of Mx to the general IFN mediated protection against viruses is not clear and secondly how Mx protein inhibit viral replication is poorly understood. The artificial up regulation of Mx transcript in mammalian and fish cell line is able to protect the cell from viral attack (Lester et al., 2012). In fish, Mx gene is triggered after viral attack but at the same time type I IFN is also stimulated which in turn induces the expression of many other antiviral proteins so the exact contribution of Mx protein to overall viral protection in fish cells is yet unknown.

The domain of mammalian Mx1, PKR and OAS1 are well studied and highly conserved in mammals (Zhou et al., 2013).
FIG. 1.5. Functions of selected IFN-inducible proteins.
Among the IFN-induced proteins believed to affect virus multiplication within single cells are PKR kinase, which inhibits translation initiation through the phosphorylation of protein synthesis initiation factor eIF-2α; the OAS synthetase family and RNase L nuclease, which mediate RNA degradation; the family of Mx protein GTPases, which appear to target viral nucleocapsids and inhibit RNA synthesis; and ADAR, which edits double-stranded RNA by deamination of adenosine to yield inosine. IFN-induced expression of MHC class I and class II antigens and NOS may contribute to the antiviral responses observed within whole animals. Ag, antigen. Adapted from reference (Samuel, 2001).
**Mx proteins and its types**

Vertebrate interferon (IFN) proteins are mediators of host defence system and are induced upon viral and microbial invasions. These proteins stimulate the expression of more than 1000 IFN stimulated genes which have antiviral, antiproliferative and immunomodulatory functions, through the Janus protein kinase- Signal transducers and activators of transcription (JAK-STAT) pathway. The three most studied IFN-induced antiviral pathways comprises of 2’-5’ OAS/RNaseL system, protein kinase R (PKR), and the Mx GTPases. Mx proteins are vital components of the IFNs antiviral defence system that belong to the dynamin family responsible for intracellular trafficking and organelle homeostasis. Mx GTPases are structurally and functionally analogous to other members of the dynamin family with their intrinsic property to self assemble and associate with intracellular membrane.

![Phylogenetic Tree of Mx Proteins](image_url)

**Fig.1.6.** A phylogenetic tree showing the different classes of Mx (adapted from Haller et al., 2015)
Mx proteins are highly conserved in vertebrates and are divided into five subgroups depending upon their similarities in sequences. Most mammals have double Mx genes that were generated after duplication event, leading to human Mx1 type and human Mx2 type (Haller et al., 2015). Interestingly, the present day mouse Mx2 is not an ortholog of human Mx2 but is similar to rodent Mx1. So the two Mx genes in the rodent subgroup are paralogous as they have risen after the gene duplication of ancestral Mx1 like gene (Haller et al., 2015). In contrast two mammals birds have 1 Mx gene while fishes have the maximum of 7 Mx isoforms that might have evolved after the Whole Genome Duplication event (Novel et al., 2013). Mx proteins are found in all IFN treated cells, are highly conserved in the vertebrate kingdom but not all Mx proteins show antiviral activity. In most of the vertebrate species Mx proteins accumulate in the cytoplasm of IFN treated cells while in rodents both nuclear and cytoplasmic Mx proteins are observed. Mx proteins have been isolated in different isoforms in the vertebrate kingdom; two genes have been reported in amphioxus and most of the mammals, including human while three in rats (Li et al., 2009). Interestingly different isoforms of Mx in the same species of fish can vary in their antiviral activity and also on antiviral specificity range (Trujillo et al., 2015).

Mx proteins were first identified in an inbred mouse strain (A2G) that showed an unusually increased level of resistance to influenza a virus (FLUAV). The resistance phenotype is present as an autosomal dominant trait that exclusively showed resistance to orthomyxoviridae family. A single gene carries the transcript of, Mx1 (for orthomyxovirus resistance gene 1), and regulated the resistance phenotype (Staheli et al., 1986; Arnheiter et al., 1990). Gene expression is usually dormant but increases after viral attack by type I or type III IFN. In humans, an ortholog of mouse Mx1 called MxA, has antiviral activity against a spectrum of viruses and the transcript is carried by Mx1 gene specific to chromosome 21 that is syntenic with mouse chromosome 16.

MxA sensitive virus includes the members of the bunyaviridae, orthomyxoviridae, paramyxoviridae, rhabdoviridae, togaviridae, picornaviridae, reoviridae and hepatitis B virus. Mx proteins are found in the entire vertebrate kingdom and the differences in amino acid sequence are responsible for the different mechanisms of antiviral activity that is peculiar for the specific strains. Recently first Mx protein was identified from disk
albalone (*Haliotis discus discus*) having properties similar to fish and mammalian Mx protein (*Zoysa* et al., 2007). Three Mx proteins were studied in gilthead seabream that showed antiviral activity against a wide range of viruses, and they also varied in their antiviral specificities (*Fernandez-Trujillo* et al., 2015). In rainbow trout (rht) also three isoforms of Mx have been found, out of which rbtMx1 and rbtMx3 are similar to Atlantic Salmon Mx1 and Mx2 respectively (*Lester* et al., 2012).

**PolyI:C and its role in viral induction**

In teleosts, the first Mx was cloned, in perch (*Perca fluviatilis*) (Staeheli, 1989) as a dsRNA induced gene. Later Mx was discovered in many fish species. Almost all viruses in the replication cycle process produce dsRNA. While dsRNA is an efficient primary signalling molecule, ssRNA is a potent stimulator of IFN response. Healthy vertebrate cells do not synthesize dsRNA <30bp in length, while viruses are known to produce longer molecules; this helps in the discrimination of self and nonself dsRNA molecules (De-Witte and Mossmann, 2010). PolyI:C is a molecule synthesized by the annealing of synthetic single stranded ployinosinic acid and polycytidylic acid that lacks 5’triphosphate residues (*Childs* et al., 2013). It is reported that polyI:C is not only analogous to dsRNA but is also an immune stimulator and toll-like receptor3 (TLR3) agonist that is frequently used as a inducer in fish to study innate antiviral responses. In Rainbow trout cell line induced with polyI:C three full length Mx cDNA were obtained by RACE PCR (*Trobridge* et al., 1997). Induction of polyI:C in Salmon was marked by upregulation of Mx transcript that showed resistance to ISAV (*Jensen* et al., 2002). Surprisingly varied expression kinetics were also seen for different Mx isoforms after VHSV infection or polyI:C induction suggesting that regulation pathways are not similar (*Tafalla* et al., 2007). Salmon infection with salmonid alphavirus-3 (SAv-3) has shown increased Mx expression (*Xu* et al., 2010). Although poly I:C is much studied immunostimulant for antiviral responses in fishes but innate immune responses differ between poly I:C and viral dsRNA which has already been elucidated in murine embryonic fibroblasts (*Poynter* et al., 2015). It has been elucidated by Poynter (2015) in rainbow trout RTG-2 cell line that IFN responses by dsRNA is limited for few hours.
but intense. However in case of polyI:C the stimulation in RTG-2 cell line is slow but longer lasting IFN and ISG transcripts have been observed.

Over-expression of Mx in rainbow trout cell did not resist IHNV infection (Trobridge et al., 1997). DNA vaccination against VHSV infection showed an increase in Mx transcript which might be a part of early protection (Mc Lauchlan et al., 2003). Mx up regulation was also observed after cycloheximide (a molecule inhibiting protein synthesis) stimulation an indication that it can be IFN independent (DeWitte- Orr et al., 2007). Larsen, 2004 reported. The antiviral activity of Mx protein of Atlantic Salmon against IPNV. Infection with nodavirus has shown up regulation of Mx transcript in the brain of *Sparus aurata* and sea bass (*Poisia-Beiro et al., 2008*). Mx homologues were also observed in zebrafish (Altmann et al., 2003), fugu (Yap et al., 2003), spotted green pufferfish (Lutfalla et al., 2003), Japanese flounder (*Ooi et al., 2006*), estuary cod (*Epinephelus coioides*) (Chen et al., 2006), catfish (*Ictalurus punctatus*) (Plant and Thune, 2008) and rare minnow (*Su et al., 2008*). The exact mechanism of these proteins is yet to be studied.

**Structure of Mx protein**

Mx is an antiviral protein belonging to the family of dynamins induced by Type I α/β IFNs in response to DNA or RNA virus and poly I: C. Proteins of the dynamin family are characterized by large molecular weight GTPases having several processes like membrane fission events, antiviral response, in plants cell plate formation and biogenesis of chloroplasts (Hinshaw 2000). Dynamins are known to have five distinct domains: an end terminal of GTPase domain, having three motifs specific for GTP-binding and a self-assembly region; a central domain having the propensity to self assemble; a pleckstrin homology domain that helps in adherence to the membrane; and the GTPase activity is initiated by coiled coil domain or th GTPase effector domain, GED and brings about self assembly of the molecule and a proline/arginine abundant region for dynamin- dynamin interactions.

Staheli et al (1986), a transgenic laboratory mice did not show any signs of infection after infected with Mx sensitive viruses even in the absence of an IFN response. Thus highlighting the antiviral nature of Mx protein. Apart from being antiviral in nature
human MxA has shown apoptotic functions (Mibayashi et al., 2002) that inhibit motility of tumor cells and its adherence to other membranes (Mushinski et al., 2009). Mx proteins form homo-oligomers that self assemble into circular helix shaped structures, which is essential for GTPase activity, protein stability and viral recognition (Fernandez-Trujillo et al., 2015). These are high molecular weight GTPases, heterotrimeric G proteins having molecular mass in the range of 70-80kDa, reduced attraction for GTP and a inherent property of GTP hydrolysis (Gao et al., 2011). Similar to the other members of the dynamin family at the N-terminal end it has a GTPase domain, in the middle a central interactive domain (CID) and an effector domain rich in leucine zipper motifs (LZ) is specific to the C-terminus. These large GTPases are IFN induced and play a vital role in controlling intracellular pathogens. The N-terminus of the Mx protein is more conserved compared to C-terminus because variable LZ motifs. The functionality of GTPase effector domain (GED) of dynamin and the GTPase activation is co-ordinated by the LZ region of the protein. In mMx1 a region of charged and basic amino acids constitutes a nuclear localization signal (NLS) (Zürcher et al., 1992). In Mx GTPases typical pleckstrin homology domain (PH) and proline /arginine rich domain (PRD) having several SH3 binding sites found in other members of the dynamin family is also lacking. Molecular modelling of human MxA protein hypothesizes it to be a ring shaped antiviral complex having GTPase domain on the outer side and the L4 loops located inwards in the ring structure (Gao et al., 2011). Structural changes in Mx proteins will not only affect the viral target binding but also its virus specificity. This might be one of the reasons for functional difference in different isoforms of Mx (Fernandez-Trujillo et al., 2015).
Fig. 1.7. Showing the 3D structure of Mx protein with stalk, hinge, Loop, GTPase and GED domain

**GTPase domain**

Mx proteins like other members of the dynamin family have a GTPase domain that is highly conserved part of the protein. In Mx protein this domain primarily consists of three GTP-binding motifs (GDXXSGKS, DLPG and TKPD) and a dynamin signature (LPRXXGXXTR) (Song et al., 2004). The dynamin signature residues combine with Mg\(^2\) ion essential for GTPase activity (Song et al., 2004). The GTP-binding motifs GDXXSGKS, DLPG are required to bind to the phosphate moiety of GTP and the third GTP-binding motif TKPD binds guanosine. The G2 and G3, GTP binding motifs are mostly conserved in all species while the first GTP binding domain is not very identical among different species. Though the dynamin signature is not totally conserved, but the penultimate threonine residue is present in all Mx proteins except turkey Mx protein, which is required for the activity of Mg\(^{2}\) ion. Due to its biological activity Mx protein have a conserved GTPase domain (Pitossi et al., 1993).
**Middle Domain or GTPase Effector Domain**

In the Mx protein structure, the GTPase domain is adjacent to the middle domain and the GTPase effector domain. Both these domain are essential for biological activity and structural conformation of Mx proteins. MD is essential for oligomerization and virus target identification (Gao et al., 2010). The GED domain has the Leucine Zipper Motif, and folds back to have an intramolecular interaction by binding with the N-terminal GTP-binding domain thus inducing the GTPase activity. The Leucine zipper at the C-terminal end is highly conserved and is essential for the antiviral activity.

**Oligomerization of Mx protein**

Mx proteins form tetramers in low protein concentration and oligomerize into large filaments and rings at higher protein concentration. These structure have been further confirmed by electron microscopy and characterized by size exclusion chromatography and sedimentation assays (Koch and Haller, 2002). The structure has three interfaces and one loop region (L4). These interfaces start an intermolecular interaction between different Mx molecules ultimately forming a ring like structure. In the rings the stalk domains are directed inwards while GTPase domains are located outwards. Thus the target viral nuclear protein is trapped in the ring like structure. It has been found that both the oligomeric structure and the GTPase activity are essential for antiviral activity. In the Mx structure the GTPase domain is directed outwards and the L4 loops responsible for antiviral activity are positioned inwards in the ring. Fernandez-Trujillo et al., (2015) have speculated that the Mx isoforms of gilthead seabream have shown different antiviral activity perhaps due to the different oligomers in each isoforms of Mx. Thus it has been reported by the above mentioned group that the structural stability as well as its functional characteristics depends upon the oligomerization of Mx protein.

**Predicted structure of Mx protein**

Gao et al., (2010) have recently predicted the crystal structure of human MxA protein that showed maximum resemblance to three -dimensional structure of dynamin proteins. In the crystal structure of human MxA, the GTPase domain is attached to the
bundle signalling element (BSE) by a hinge, and the BSE is connected to the stalk by a second hinge. The GTPase domain consists of a central β sheet surrounded by α-helices (Judith et al., 2013). BSE is composed of three α-helices, first helix consists of amino acids preceeding the GTPase domain, second helix is in between the GTPase domain and MD and the third is at the C-terminal part of the GED. The C-terminal leucine zipper and three helices from the MD congregate to form a helix rich structure called the stalk (Paolo et al., 1999). Though the human MxA is the only Mx protein crystallized so far, it shows maximum structural similarity to the members of the dynamin family and other large GTPases.

**Structural basis of Mx function**

Some vital functions of Mx protein associated with its self assembly and oligomerization is GTPase activity, protein stability and recognition of viral target structures. When one molecule of Mx binds to CID of a second neighbouring molecule then oligomers are formed. Experimental analysis have shown that an exchange in amino acid position 612 from leucine to lysine of MxA, has changed its basic property of self assembly and GTP hydrolysis signifying that oligomerization is essential for GTPase activity(Janzen et al.,2000). Further, Janzen have also revealed that this mutated form of Mx is easily degraded in contrast to the wild form whose half life is more than 24hrs. Thus it can be speculated that the oligomerized form of Mx is essential for both its functional activity and structural stability for prolonged periods. Some knowledge on the functional aspects of Mx protein can be known from the structure other members of the GTPases. In mouse Mx1 it was found that by truncating by 19 amino acids from C-terminal region or by replacing 30 amino acid sequences from the inactive Mx2 it lost its antiviral activity against influenza. However replacing the arginine at 614 position by glutamic acid, or replacing the end terminal 30 amino acid sequence with that of human MxA did not change its antiviral nature once it was located into the nucleus. The vital role of C-terminus end of Mx protein in mediating the antiviral function can also be elucidated from the fact that inactive rat Mx3 differs from rat Mx2 by a sequence of eight amino acid in the C-terminus.
Mitchell et al (2012) have found that certain selected residues in loop L4 of MxA mediates the antiviral role of Mx. They have shown that primate antiviral activity is dependent on the loop L4 especially for orthomyxoviruses THOV and influenza A. The hinge connecting the CID and effector domain are probably connected by a proteinase K-sensitive hinge (Haller et al., 2007). Point mutations in the LZ region of MxA and rat Mx2 showed that the C-terminal effector domain is prerequisite for identification of viral targets (Züürcher et al., 1992). Members of this family are present in different cellular locations where they different functions such as intracellular vesicle transport, endocytosis and mitochondria distribution. In particular, the GTPase domain at the N-terminal end of Mx protein has a highly conserved of <300 amino acids, a central domain of <150 amino acids and the C-terminal end is the GTPase effector domain (GED) of <100 amino acids comprising of two leucine zippers that have an inherent property to form alpha helices. The Mx structure is a helical ring in which GTPase domain are at the periphery, stalk assemble in the middle to form a crisscross pattern and loopL4 facing outward from the inner surface(Gao et al.,2011). This configuration allows multiple attachment site between the Mx and its substrate viral nucleoprotein. Thus slight differences in the binding affinity between MxA and viral nucleoprotein determines the antiviral specificity.

**Mx action and its intracellular localization**

The antiviral properties of Mx proteins differ depending upon its intracellular localization of the individual proteins as well as on the type of virus. Several reports highlight the direct interaction of Mx protein and its viral target. The human MxA can inhibit influenza virus multiplication both in the nucleus and cytoplasm. However mouse Mx1 is active against the influenza virus only in the nucleus but not in the cytoplasm. A mutant mouse Mx1 protein lost its NLS signal, is active once it is transported to the nucleus. The nuclear forms (Mx1 in mouse, rat and cotton rat) show antiviral activities against virus that replicate in the nucleus like the orthomyxoviruses FLUAV and togoto virus THOV. Similarly cytoplasmic Mx proteins inhibit viruses that multiply in the cytoplasm like the rhabdoviruses (e.g. vesicular stomatitis virus, VSV) and bunyaviruses (e.g. LaCrosse virus, LACV). In humans MxA and MxB are two Mx
proteins localized in the cytoplasm out of which MxB does not show antiviral activity. MxA is effective against a wide range of viruses including the bunyaviruses, orthomyxoviruses, paramyxoviruses, rhabdoviruses, togaviruses, picornaviruses, and Hepatitis B virus, a DNA virus with a genomic RNA intermediate (Haller, 2007). In fish up regulation of Mx expression has shown resistance to IPNV, ISAV and sole aquabirnavirus in the Chinook Salmon embryonic cell line, reovirus in rare minnow, Hirame rhabdovirus and VHSV in Hirame Natural Embryo cell line, Yellow Grouper Nervous Necrosis Virus in grouper brain 3(GB3) cells and nodavirus Grouper fin cells (Lester et al., 2012). However it does not provide protection against IHNV in CHSE cells or against iridovirus in barramundi cell line.

**Antiviral activity of Mx protein**

The exact mechanism of antiviral action of Mx protein is not clearly understood. However the mode of action of Mx protein to prevent viral replication is not the same for all the viruses. Thus briefly their mechanisms against few viral families have been described.

**Antiviral activity against Orthomyxovirus**

The reference virus is the influenza virus and antiviral properties of Mx protein were first reported against this virus in mouse. The influence of mouse Mx1 virus on different stages of influenza virus replication has been comprehensively studied, yet the exact antiviral mechanism is poorly understood. Mouse Mx1 does not affect either the uncoating of the virus or trafficking of ribonucleoprotein into the nucleus. Further Mx1 expressing cells after IFN stimulation lowers primary viral transcription and viral mRNA translation (Meyer and Horisberger, 1984). Again different Mx pathway was followed in different cell such as in IFN treated macrophages Mx1 mostly inhibits viral translation while IFN treated embryonic fibroblasts affected mostly primary transcription. Mx1 inhibits the transcription of the different influenza viral proteins. The larger transcripts (encoding PB1, PB2 and PA) are easily inhibited, hemagglutinin (HA) transcripts are moderately inhibited and NP transcripts but rarely affects the transcription of M and NS segments. Mouse Mx1 targets the influenza viral Ribonuclear proteins (vRNPs). Two subunits first PB2 protein subunit and its presence
can inhibit Mx1 activity. The second is the NP protein, which determines the viral activity of different strains of influenza A (Verhelst et al., 2012). Three residue positions are grouped in overlapping region in the body domain of NP protein that is responsible for Mx sensitivity. GTPase activity is essential for antiviral activity but surprisingly NP and PB2 interactions with Mx1 is not dependent upon GTPase activity (Meyer and Horisberger, 1984). Thus from the available data it can be assumed that Mx1 attacks the NP interface of vRNPs leading to the disruption of the vRNPs thus inhibiting the spread of viral infection. Human MxA inhibits the viral primary transcription before viral protein expression of influenza virus genes similar to mouse Mx1. The human MxA protein, impedes the replication of influenza virus within the cytoplasm as well as the nucleus, while the mouse Mx1 protein is active only inside the nucleus. Activation of mouse Mx1 in the nucleus probably might be due to the recognition of viral targets that adopt the desired conformation only in the nucleus. Human MxA blocks the vRNPs by forming a self assembled ring around these vRNPs. In case of pig Mx1 acts on the endocytic pathway and delays the entry of vRNPs into the nucleus (Palm et al., 2010). Infectious Salmon Anaemia Virus (ISAV) belonging to the genus *Isavirus* and family *Orthomyxoviridae*. This virus is enveloped with negative sense RNA having surface projection of haemagglutinin surface esterase protein and a separate putative fusion protein. As cited earlier human and mouse Mx1 inhibits different stages in the influenza viral replication cycle, so it can be speculated that Atlantic Salmon Mx protein located in the cytoplasm will also interfering with the replication cycle of ISAV as the exact mechanism is not known.

**THOV**

Thogoto virus (THOV), is a native pathogen of mice (Koch and Haller, 1999) is also a member of the *Orthomyxoviridae* family. Human MxA inhibits the transfer of vRNPs into the nucleus by blocking the RNA-bound NP that ultimately stops the signals responsible for nuclear translocation of the vRNPs (Koch and Haller, 1999; Weber et al., 2000). The C terminus end of human MxA has loop L4 that interacts with the viral NP. Human MxA and mouse Mx1 are known to restrict THOV polymerase activity in the nucleus but does not impede vRNP transport (Haller et al., 2015).
**Antiviral Activity against Rhabdovirus**

Cytoplasmic Mx proteins are known to inhibit the multiplication of Vesicular stomatitis virus (VSV). MxA prevents VSV mRNA synthesis, by inhibiting elongation of viral RNA chain. GTP catalyzes the inhibition of VSV transcription by purified MxA and also nonhydrolyzable GTP analogs (Schwemmle et al., 1995). Thus substantiating the fact that GTP binding is essential for antiral actiy and can done without GTPase activity. It is being speculated that MxA inhibits vRNP of VSV but an interaction between human MxA and VSV proteins have not been demonstrated. Two isoforms of Mx protein isolated from Japanese flounder (JF) which are active against Hirame rhabdovirus (HIRRV) and Viral haemorrhagic septicaemia virus (VHS) (Rhabdoviridae) and the Viral haemorrhagic septicaemia virus (VHS) (Rhabdoviridae) have been studied by (Caipang et al., 2003). Linear negative sense RNA encoding six viral genes namely, nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), nonviral protein (NV), and RNA polymerase (L) are specific to hirame rhabdovirus (HIRRV) and the Viral haemorrhagic septicaemia virus (VHSV) of the genus *Novirhabdovirus* (Walker et al., 2000). In JF-Mx expressing cells titer of rhabdoviruses were lower than the infected and control cells. Kinetics of the rhabdoviral expression, showed decreased level of viral glycoprotein and nucleoprotein thus indicating that JF-Mx interferes with later stages of rhabdoviral replication.

**Antiviral activity against Bunyavirus**

Members of the *Bunyaviridae* are inhibited by human MxA and Mouse and Rat Mx2 proteins. The prevention of replication of La Crosse virus (LACV) virus is done by sequestering the newly produced viral nucleocapsid by forming membrane associated perinuclear complexes. The incomplete structural conformation does not allow nucleocapsid protein to carry on viral replication. Active viral RNA polymerase is the target of MxA by inhibiting the accumulation of viral transcripts (Reichelt et al., 2004).
**Antiviral activity against Birnavirus**

IPNV (Infectious Pancreatic Necrosis Virus) is a double stranded RNA nonenveloped, icosahedral virus, that belongs to genus Aquabirnavirus and family Birnaviridae. IPNV is a highly contagious, widely affecting farmed and cultured fish. It causes mass mortality and survivors become asymptomatic carriers. It has been shown by Fernandez and Trujillo that all the three isoforms of sea bream Mx (Sau) have different pathway for IPNV inhibition. Sau Mx1 inhibits the synthesis of viral VP1 RNA, without affecting viral multiplication; Sau Mx2 decreases the viral yield by interacting with viral protein finally SauMx3 reduces the synthesis of the polyprotein RNA, thus preventing viral multiplication. Thus it can be said that all the Sau Mx work concomitantly to reduce viral replication. The VP2 protein in IPNV virus determines its virulence by having a certain residues in the domain that plays an eminent role in adsorption and epitope recognition (Coulibaly et al., 2005). Depending upon the position of residues the virulent or avirulent nature of virus is determined. It was observed that the 217, 221 and 247 residues of VP2 influence the replication index of IPNV. The strains that have Pro-217 and Ala-247 as was the case with V70 and V98 have a higher number or are more virulent compared to those viruses with Thr-217 and Thr-247 (Song et al., 2005). In cells that are either treated with IFN or treated with cytokine at the immediate outset of IPNV infection, replication of vRNP is inhibited, however in already infected cells Mx expression is unable to inhibit viral replication (Skjesol et al., 2009). Again the level of induction of Mx expression and also the antiviral activity is different depending upon the different strains of IPNV. In vivo injection of IPNV into S.salar posts smolt induces the expression of Mx3 but it cannot prevent infection (Jensen and Robertsen, 2000). Baramundi Mx is potent inhibitor of the proliferation of fish nodavirus and birnavirus (Chen et al., 2005). Cells expressing recombinant Senegalese sole Mx, gilt-head seabream Mx or turbot Mx prevent the manifestation of Sole aquabirnavirus (Garcia et al., 2008).

**Antiviral activity against Nodavirus**

Viral Nervous Necrosis (VNN) is a severe disease affecting larvae and 19 fishes of ten families causing mass mortalities worldwide. VNNV belongs to the nodaviridae family.
Nonencapsulated nodaviral genome possesses two single-stranded positive sense RNA. RNA1 has gene specific to RNA-dependant RNA polymerase (RdRp) and RNA2 gene is encrypted with the coat protein (CP). The symptom of the infection is characterized by vacuolation and necrosis of the central nervous system in host (Chen et al., 2008). Mx protein of grouper directly binds with coat protein of nodavirus and regulates viral multiplication. The coat protein has affinity for the effector domain of Mx. The grouper Mx reduces viral titer by inhibiting both coat protein and RNA-dependant RNA polymerase of nodavirus antigens.

**Antiviral Activity against Paramyxovirus**

Human MxA are known to be effective against a few viruses of *Paramyxoviridae* family. Depending upon the cell type its antiviral activity can be controlled. The inhibition of Measles virus multiplication in the human mononuclear cell line U937 and in the glioblastoma cell line U87 by human MxA but not in Vero cells (Schnorr et al., 1993).
Fig. 1.8: The antiviral Mx pathway in the life cycle of various viruses.
(A) Human MxA blocks the trafficking of viral ribonucleoprotein complex of FLUAV and THOV into the nucleus thus inhibiting secondary transcription and replication. (B) MxA inhibits the replication of VSV in the cytoplasm. (C) The N protein of Bunyavirus
needed for replication is sequestered by MxA thus blocking replication viral genome. (D) The stability of viral nucleoprotein is disturbed; its nuclear uptake is inhibited preventing integration of proviral DNA.( adapted from Haller et al.,2015).

**Table 1.3: Different types of Mx protein, its localization and effect on different virus**

<table>
<thead>
<tr>
<th>Mx protein</th>
<th>Localization</th>
<th>Antiviral activity</th>
<th>Virus Family</th>
<th>Genome</th>
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<td>THOV</td>
<td><em>Orthomyxoviridae</em></td>
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<td></td>
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<td><em>Bunyaviridae</em></td>
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<td>Rabies virus</td>
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<td>VSV</td>
<td><em>Rhabdoviridae</em></td>
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Dog Mx1, Mx2  
Cytoplasm  
VSV  
Rhabdoviridae  
ssRNA (+)

Atlantic salmon Mx1-Mx3  
Cytoplasm  
ISAV  
Orthomyxoviridae  
ssRNA (-)

Japanese flounder Mx  
Cytoplasm  
HIRRV  
Rhabdoviridae  
ssRNA (+)

Grouper Mx1-Mx3  
Cytoplasm  
YGNNV  
Nodaviridae  
ssRNA (+)

Senegalese sole Mx  
Cytoplasm  
Aquabirnavirus  
Birnaviridae  
dsRNA

Barramundi Mx  
Cytoplasm  
NNV  
Nodaviridae  
ssRNA (+)

IPNV-SP  
Birnaviridae  
dsRNA

Seabream Mx1-Mx3  
Cytoplasm  
IPNV  
Birnaviridae  
dsRNA

Rare minnow Mx  
Cytoplasm  
GCRV  
Reoviridae  
dsRNA

Rainbow trout Mx1  
Cytoplasm  
IPNV  
Birnaviridae  
dsRNA

SAV  
Togaviridae  
ssRNA (-)

Other functions of Mx proteins

All Mx proteins are not antiviral in nature eg. Human MxB, Rat Mx3, duckMx, dog Mx1 (Nakamura et al., 2005) and many teleost Mx proteins except for Atlantic Salmon and Japanese flounder. Like the dynamins Mx proteins may also have many cellular functions (Haller and Koch, 2002). Up-regulated human MxA carried out transferrin endocytosis (Jatiani and Mittal, 2004) also self assembled into ring like structures which tabulated lipids in vitro (Accola et al., 2002). Human MxB is also reported to play a vital role in nuclear import and endocytosis (King et al., 2004). Mx proteins are also expressed in endometrium of sheep, cows, gilt, mare, mice and women (Hicks et al., 2003).
AIM OF THE THESIS

Objective of the study

- Isolation, cloning and expression of Mx gene from *Cirrhinus mrigala*.
- Peptide synthesis and raising of polyclonal antibody against the Mx protein
- Studying anti-viral, anti-bacterial and anti-parasitic responses of Mx proteins
- To investigate the expression of Mx-protein in tissues including blood cells of *Cirrhinus mrigala* after experimental induction of the protein by poly I: C treatment/ viral induction.

Objective 1: Isolation, cloning and expression of Mx gene from *Cirrhinus mrigala*

- Mx expression will be studied in different tissues (kidney, liver, and spleen) of *Cirrhinus mrigala* after 24 h of induction of PolyI:C.
- RT-PCR after PolyI:C stimulation will be done to evaluate constitutive Mx expression in kidney, liver, heart, brain, gill, muscle, intestine, blood and spleen.
- Mx gene fragments obtained after PCR will be cloned into pGEMT vector and sent for sequencing.
- Through primer walking and RACE PCR, 5’ and 3’end full length Mrigal Mx CDS will be obtained.

Expression kinetics

- Constitutive expression of Mx will be studied in brain, heart, spleen, kidney, liver, muscle, gill, intestine and blood of Mrigal for fourteen days post induction.
- Through the semi-quantitative PCR method presence of Mx transcript will be detected
- Absolute quantification of Mx expression in different tissues of Mrigal will be done by RT-PCR
Objective 2: Peptide synthesis and raising of polyclonal antibody against the Mx protein

- A peptide fragment will be designed from the conserved sequence of Mrigal Mx protein and other available Mx protein in the database.
- The peptide sequence will be procured from Sigma.
- This fragment will be injected to rabbit and antiserum raised in rabbit for further experiment.
- Western blot and immunocytochemistry will be done.


- Expression of Mx transcript in different tissue of Mrigal will be studied after OmpC vaccination.
- Presence of Mx gene will be analysed in *A. hydrophila* challenged Mrigal.
- Immunomodulatory nature of Mrigal Mx protein will be evaluated after parasitic infestation.

Objective 4. To investigate the expression of Mx-protein in tissues including blood cells of *Cirrhinus mrigala* after experimental induction of the protein by poly I: C treatment/viral induction.

- Smears of blood and buffy coat layers will be immunoassayed for Mx protein.
- Protein content of different tissue will be analysed by protein profiling through SDS PAGE.
- Western blot analysis for plasma and tissue extracts will be done.