Indian Journal of Animal Sciences **87** (6): 668–679, June 2017/Review

Apolipoproteins in fish: From lipid transport to innate immunity

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Received: 13 October 2016; Accepted: 8 November 2016

ABSTRACT

The fish has a strong innate immune system, and antimicrobial peptides play a major role in fish innate immunity, providing potential defence against broad spectrum of fish pathogens. Apolipoproteins,that are abundant proteins of plasma, playing important role in lipid transport and metabolism, also have potential antimicrobial activity. The present review describes the classes, structural details and important biological functions of apolipoproteins reported in both mammals and fish with an emphasis on their roles in host defence. The role of fish apolipoprotein A-I, a major component of high-density lipoproteins (HDL), is described in great detail using different infection models along with its bactericidal and immunomodulatory activities in various fish species against wide range of fish pathogens.Further, role of some novel fish-specific apolipoproteins, including the mammalian ones, have also been defined with a special focus on the molecules described in Indian carp species. As the understanding on major apolipoproteins is limited in fish species, this review might serve as a foundation to explore further their functional diversity in Indian fish species.

Key words: Apolipoproteins, Antimicrobial properties, Fish, Innate immunity

All organisms are exposed to a wide array of pathogens in their day to day life. However, they do not contract from disease frequently due to their potent innate immune effectors. Innate immune components of higher vertebrates are mainly inherited from invertebrates in the process of evolution.Innate/non-specific immunity consists of many cells, viz. monocyte-derived macrophages, dendritic precursor cells, natural killer cells, neutrophils, eosinophils, mast cells,basophils and epithelial cells. Pattern recognition receptors (PRRs) present on these cells help to recognize pathogen associated molecular patterns (PAMPs). PRRs include molecules like C-type lectins, macrophage scavenger receptors, pentraxins, leucine-rich proteins, lipid transferase, inflammasome proteins and integrins. Pathogen recognition helps in activation and production of antimicrobial peptides (AMPs), cascade of complements, cytokines, differentiation and maturation of dendritic cells leading to antigen presentation and finally activating adaptive immunity of an organism (Kaji 2013). The fish has a strong innate immune system, and primary interference to pathogen occurs through mucus layer that covers its entire body surface. This mucus layer mainly contains AMPs, which are secreted by epithelial cells and act as the first line of defense against microbial invasion (Ganz 1999). AMPs are small unique molecules (15–100 amino acids) that display broad spectrum antimicrobial activities against bacteria, fungi, virus and parasites along with playing role in inflammatory responses (Johnston *et al*. 2008). They recruit neutrophils and fibroblasts, promote mast cell degranulation, enhance phagocytosis, decrease fibrinolysis and prevent tissue injury, and promote apoptosis of infected cells (Hancock and Diamond 2000, Zasloff 2002, Boman 2003). HDLs (high-density lipoproteins), abundant plasma proteins of mammals which mainly take part in reverse lipid transport, also have some immune-related properties like antimicrobial activity, regulation of inflammatory cytokines, anti-atherosclerotic activity, anti-oxidant activity and anti-thrombotic activities (Villarroel *et al.* 2007, Kaji 2013, Qu 2014). HDLs mainly efflux cholesterol to liver from peripheral tissues for metabolism and excretion. Surprisingly, only one-third of HDLs take part in lipid transport and metabolism. Other two-third of HDLs contributes towards immune responses in organisms (Gordon *et al.* 2011).

Infectious diseases are the major constraints in the development and sustainability of aquaculture sector leading to losses in the production and trade, and the causative agents include various pathogens, viz. viruses, bacteria, fungi, parasites and other undiagnosed and emerging pathogens. Use of antibiotics can lead to development of drug-resistance variety of pathogens, and vaccination for fish diseases is still now in infancy, particularly in Indian subcontinent. The alternative method for protection from diseases, is use of immunostimulants,

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which can provide immediate protection from wide range pathogens. As fish use lipids rather than carbohydrates as their main energy source, lipid metabolism and lipoprotein physiology are very important for their homeostasis (Kondo *et al*. 2005). Apolipoproteins play important role in innate immunity and produce inflammatory response. Among all apolipoproteins, antimicrobial and immunomodulatory roles of apolipoprotein A-I and A-II were extensively studied in different fish species. Other apolipoproteins mostly play major role in organogenesis, embryonic development and protection, food intake, protease inhibition, complement regulation and acute phase response. These broader functions of apolipoproteins and their structural heterogeneities make them interesting molecules to be studied during different infection models for aquatic diseases.In recent years a lot of literature has been generated on a few fish species with regards to biological functions of this novel class of molecules. This review is an attempt to compile the work done on different fish species targeting various broad ranges of apolipoproteins, emphasizing their prominent role during immunity with relation to their structural diversity in comparison to their mammalian counterparts.

Types of apolipoproteins in human and fish species

Apolipoproteins are the protein moieties of lipoproteins that play critical role in lipoprotein metabolism (Paolucci *et al*. 1998). These lipid binding proteins function as the structural backbone of lipoprotein particles and regulate cellular lipid flux through their interaction with cell surface receptors (Otis *et al*. 2015). They are classified according to size, density, lipids and apoprotein composition. There are 6 major classes of apolipoproteins, viz. A, B, C, D, E and H. HDLs constitute of 50% apolipoproteins, mainly AI, AII, CI, CII, CIII and E. Most of these apolipoproteins are being well characterized in humans and a few of them have been functionally characterized in various fish species. The different classes of apolipoproteins studied in humans and fish species and their brief functional details are given in Table 1.

Apolipoprotein A

This group of lipoprotein contains 3 different types of subgroups, A-I, A-II and A-IV. Apolipoprotein A-I (ApoA-I) is the major component of HDLs that plays important role in lipid metabolism and innate immunity. ApoA-I is a single polypeptide containing 243 amino acids in human and 250–260 amino acids in different fish species. It contains a signal peptide of 17 amino acids in all most all organisms which indicates its well conserved sequence during the process of evolution. This plasma protein is mainly synthesized by liver tissue. ApoA-I possesses a highly flexible structure which allows it to exist in different states: lipid poor, lipid free and discoidal or spheroidal lipoproteins of different sizes (Bashtovyy *et al*. 2011). This protein contains two independently folded domains, one is C-terminal domain which is loosely structured and show high affinity for lipid binding, and second is N-terminal helix bundle domain that acts as lipid anchor (Jenssen *et al*. 2006, Kaconis *et al*. 2011). The c-terminal domain has an ability to bind the lipid component of bacterial cell membrane making it antimicrobial in nature. Along with that, extensive structural analysis revealed that it also contains large hydrophobic regions and high cationic residues in its amphipathic α-helix motif, which are the signature feature of potential antimicrobial proteins (Beck *et al*. 2013).ApoA-I also possesses specific anti-inflammatory properties as it inhibits lipid peroxidation, monocyte activation and produces cytokine by interaction between T cells. Again recently, Vuilleumier *et al.* (2013) reported that this anti-inflammatory molecule can also act as pro-inflammatory one upon post-translational modifications. Almost all fish ApoA-I which are being characterized so far,have antimicrobial properties because they possess these structural features. In one of the Indian major carp species, rohu (*Labeo rohita*),and an array of functional domains of ApoA-I have been identified and well characterized (Mohapatra *et al*. 2016). It contains a Pfam domain that mainly helps in lipid binding and being present in all most all exchangeable apolipoproteins and their multigene members. HTH_ICLR (helix_turn_helixisocitratelyase regulation) functions as a DNA-binding protein region that binds through helix-turn-helix motif. The membrane attack complex/perforin (MACPF) domain mainly helps in pore formation and is conserved in most of the innate and adaptive immunity molecules. The presence of CUE domain (coupling of ubiquitin conjugation to ER degradation domain) involves in binding ubiquitin-conjugate enzymes. Helix loop helix domain (HLH domain) contributes towards antimicrobial activity by pore formation in inner membrane of bacteria. All these domains further contribute towards antimicrobial property of *L. rohita* ApoA-I (Mohapatra *et al*. 2016). A 3D model structure of *L. rohita* ApoA-I, given in (Fig. 1) shows the α-helix bundle, a remarkable feature of AMPs protein of *L. rohita.* The pink portion indicates α-helix of the protein that contributes towards the antimicrobial property.

Apolipoprotein A-II is the second major lipoprotein of

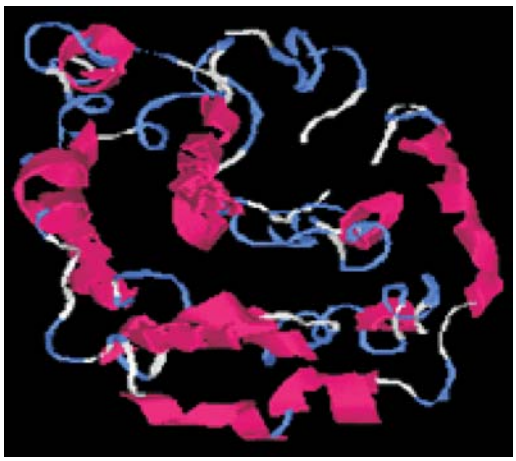

Fig. 1. 3D modeling structure of ApoA-I.

Table 1. Classification and properties of major plasma apolipoproteins from different fish species

Apolipo-proteins	General Functions	Functions characterized	Identified in species	Reference
ApoA-I	Cofactor of lecithin-cholesterol acyl transferase (LCAT), reverse cholesterol transport, sperm motility, capacitation, acrosome reaction, lipid metabolism	Antimicrobial, Immunostimulant Regeneration of optic nerve,protection to sperm and reproductive tissue from microbial attack, maintenance of sperm membrane integrity	*Ameiurus nebulosus, Chrysemys picta,Sparus aurata, Raja erinacea, Gadus morhua* L., *Anguilla japonica,Oncorhynchus , mykiss Cyprinus carpio, Danio rerio, Epinephelus akaara, Morone saxatilis, Labeo rohita*	Kaconis *et al*. (2011), Jenssen *et al*. (2006), Mohapatra *et al*. (2016)
ApoA-II	Lipid transport	Antimicrobial, Immunostimulant, protection to sperm and reproductive tissue from microbial attack, maintenance of sperm membrane integrity	*Anguilla japonica, Epinephelus coioides, Carassius auratus gibelio, Hemibarbus mylodon,* pufferfish	Irshad and Dubey (2005), Choudhary *et al*. (2009)
ApoA-14 (Specific to fish)	Not identified	Antimicrobial, of digestive system, morphogenesis organogenesis of liver and brain tissues, embryonic development	*Danio rerio, Carassius auratus gibelio, Fundulus heteroclitus,Hemibarbus mylodon,Oplegnathus fasciatus*	Kondo *et al*. (2001), Kondo *et al*. (2005), Zhou (2005), Gordon *et al.* (1985)
ApoA-IV	Activates LCAT	Regulate food intake	*Danio rerio*, *Hemibarbus mylodon*	Silvia *et al*. (2001), Otis *et al.* (2015)
ApoB-48	Lipid transport	Function unknown	*Danio rerio*	Scott (1990)
ApoB-100	Cholesterol transport from liver	Function unknown	*Danio rerio*	Scott (1990)
ApoC-I	Activates LCAT	Function unknown	*Danio rerio, Sparus aurata, Hemibarbus mylodon*	Li *et al*. (1988), Jong *et al*. (1999), Kim *et al*. (2009)
ApoC-II	Activates lipoprotein lipase, help in lipoprotein metabolism	Function unknown	*Hemibarbus mylodon*	Law *et al*. (1986), Shen *et al*. (2000)
ApoC-III	Inhibit action of Apo C-II	Function unknown	Not detected in any fish species	Lusis (1988)
ApoD	Neurons development during embryogenesis	Function unknown	*Takifugu rubripes*, *Danio rerio,Tetraodon* pufferfish,medaka	Aspinall *et al.* (1995), Terrisse *et al*. (1988), Ganfornina *et al*. (2005)
ApoE	Lipid homeostasis, cholesterol transport, control neuron associated diseases	Marker for aging, morphogenesis and organogenesis of brain	*Danio rerio, Oncorhynchusmykiss, Scophthalmus maximus, Hemibarbus mylodon*	Durliat *et al.* (2000), Poupard *et al.* (2000), Kondo *et al.* (2005)
ApoH	Anti-coagulation activity	Antimicrobial	*Labeo rohita*	Das *et al*. (2014)
ApoM	Lipid transport	Antimicrobial	*Danio rerio*, puffer fish, *Hemibarbus mylodon*, *Labeo rohita*	Karlsson *et al*. (2006), van Dijk *et al*. (2006), Mishra *et al*. (2011)
ApoJ	Promotion or inhibition of apoptosis, tissue remodeling, membrane recycling, cell-cell and cell-substratum interactions, sperm maturation, lipid transportation and complement inhibition	Function unknown	Not detected in any fish species	de Silva *et al*. 1990, Trougakos and Gonos (2002)
ApoO	Lipid transport	Function unknown	*Gobiocypris rarus*	Lamant *et al*. (2006)
ApoL	Inflammatory response, anti-parasitic, role in innate) immunity	Function unknown	*Gobiocypris rarus*	Monajemi *et al*. (2002), Sana *et al*. (2005), Smith and Malik (2009)

plasma containing 2 identical polypeptides joined by disulphide bond at position 6 (Irshad and Dubey 2005). This protein has been isolated from human, horse, pig, chimpanzee and common carp, *Cyprinus carpio* (Choudhary *et al*. 2009), being synthesized in liver. In fish, a 14 kDa apolipoprotein A-II has been described in Japanese eel, pufferfish and orange-spotted grouper (Kondo *et al*. 2001, Kondo *et al*. 2005, Zhou 2005). In gibel carp this protein has been reported to have role in early larval development and organogenesis of digestive system during embryogenesis (Xia *et al*. 2008). ApoA-14 kDamolecule of fish has homology to ApoA-II of mammals because of their greater sequence similarities in terms of internal repeats in both sequences and close clustering in the phylogenic analysis (Choudhary *et al*. 2009). Hence, ApoA-14 kDa was termed as ApoA-II like protein. However, the fish sequence lacked propeptide as of higher vertebrates, and its mature peptide is secreted to plasma without cleavage of signal peptide (Gordon *et al*. 1985). Like ApoA-II of mammals and chicken, the N-terminal domain sequence of ApoA-14 kDa was identified in rainbow trout (*Oncorhynchus mykiss*), zebrafish (*Danio rerio*), gibel carp (*Carassius auratusgibelio*), killifish (*Fundulus heteroclitus*), Korean dotybarbel (*Hemibarbus mylodon*) and striped beakfish (*Oplegnathus fasciatus*) (Choudhary *et al*. 2009). It is worth looking into the structural and functional details of similar molecule, if at all existing in Indian carp species, a widely culture fish.

Apolipoprotein A-IV is mostly synthesized in intestine, secreted to plasma, and activates enzymes like lecithin-cholesterol acyl transferase (LCAT) (Silvia *et al*. 2001). This protein has been reported in zebrafish that regulates fat food intake, similar to mouse ApoA-IV (Otis*et al*. 2015). Not much has been explored into its existence and functional diversity in other important fish species.

Apolipoprotein B

This group contains two plasma proteins, ApoB-100 and ApoB-48,being synthesized by a single gene. The gene present in liver synthesizes ApoB-100 which is present along with other apoproteins in VLDL, IDL and LDL, whereas the same gene in intestine codes for a smaller protein,ApoB-48 because of C-terminal truncation. The mechanism behind the synthesis of two different proteins from the same gene is not well understood (Scott 1990). ApoB is only reported in zebrafish till date and in phylogenetic analysis it shares a same clade with human ApoB. As compared to human ApoB subgroups, zebrafish also have 3 types of ApoB proteins,ApoBa, ApoBb.1 and ApoBb.2. The gene organization in liver and heart tissues produces ApoBaand ApoBb.1 is being produced by intestine as in human and mice. ApoBb.2 is the shortest isoform without truncated C-terminal, except numerous deletionsbeingobserved throughout the protein sequence. ApoBb.1 mostly plays a greater role in lipid transport but the mechanism behind the production of different isoforms is still not clear (Otis *et al*. 2015). Recently, ApoB has also been described in rohu, although its detail characterization is yet to be carried out (Robinson *et al*. 2012).

Apolipoprotein C

This group contains 3 types of proteins ApoC-I, ApoC-II and ApoC-III which are surface components of chylomicrons, very low-density lipoproteins (VLDL) and HDL (Li *et al*. 1988, Jong *et al*. 1999). ApoC-I is a small polypeptide which activates LCAT (7 IJBB). ApoC-I has been detected in zebrafish, *Sparus aurata* and *Hemibarbus mylodon*. Tissue-specific study of *H. mylodon* Apo C-I reveals a strong level of expression in liver tissue besides its presence in all the tissues (Kim *et al*. 2009). ApoC-II is a single chain polypeptide which activates lipoprotein lipase (LPL) and helps in lipoprotein metabolism (Law *et al*. 1986). High expression of ApoC-II transcript has also been observed in most of the tissues including liver in *H. mylodon*. The ApoC-II of rainbow trout also helps in lipid metabolism by activating LPL in temperature-dependent manner. In low temperature (around 10°C), a remarkable increase in LPL activity was being observed which might be indicative of adaptation mechanism towards restricted lipid metabolism at lower temperature (Shen *et al*. 2000, Kim *et al*. 2009). ApoC-III contains an oligosaccharide chain of one galactose, one galactosamine and residue of sialic acids (Lusis 1988). It has inhibitory action against ApoC-II (Calvert and Abbey 1985, Sleisanger and Fordtran 2002).

Apolipoprotein D

ApoD is having a glycosylated polypeptide which has no structural similarity to other apoproteins (Chen *et al*. 1987, Drayna *et al*. 1987). This protein is found in HDL along with LCAT and ApoA-I in plasma.It is also present in blood, liver, intestine and brain (Calvert and Abbey 1985, Lusis 1988, Navarro *et al*. 1988).This protein helps in transport of small hydrophobic ligands and acts as a marker molecule for diagnosis of female prostate cancer, schizophrenia, malignant melanoma and Alzheimer disease in human but exact function of this protein is still unknown (Aspinall*et al*. 1995, Terrisse *et al*. 1988). It has been detected in *Tetraodon* pufferfish, *Fugu* pufferfish, zebrafish and medaka, and phylogenetic analysis reveals that fish ApoD supports an ancestral origin of ApoD in chordate lineage (Ganfornina *et al*. 2005).

Apolipoprotein E

This protein is mainly associated with plasma proteins that help in lipid homeostasis and cholesterol transport in and out of cells by mediating phospholipid cholesteryl and ester triacylglycerol (TAG)(Mahley and Huang 1999, Mahley and Rall 2000).ApoE is synthesized in liver and macrophages of peripheral tissues, and by astrocytes of brain and spinal cord (Bu 2009). ApoE mainly acts as antioxidant against lipid oxidation, modulator of neurotropic factor and plays an important role in transportation of lipids to neurons via ApoE receptors. Production and release of

ApoE depends on cytokine immunomodulation. The pro-inflammatory cytokines decrease production of ApoE whereas anti-inflammatory cytokines promotes production of this protein. It plays an important role in maintenance of the balance between T cell subgroups (Th1/Th2) and activation of macrophages (Zhang *et al*. 2011). This also acts as marker molecule for various old-age related diseases like atherosclerosis (Davignon *et al.* 1988, Mahley and Rall 2000, Lahoz*et al*. 2001), Alzheimer's disease (Corder *et al*. 1993, Roses 1996) and Parkinson's disease (Li *et al*. 2004). Life longevity of human is associated with varied expression of this specific apolipoprotein (McKay *et al*. 2011). ApoE has been reported in several fish species, viz. zebrafish (Babin*et al*. 1997), rainbow trout (Durliat *et al.* 2000), turbot (Poupard*et al*. 2000), pufferfish (Kondo *et al*. 2005), spotted barbell (Kim *et al*. 2009) and rohu (Robinson *et al*. 2012).This amphipathic helical protein possesses 2 independently folding domain, a cluster of basic amino acid residue N-terminal domain helping in receptor binding activity (Thomas *et al*. 2005) and lipid binding C-terminal domain (Wilson *et al*. 1991). In lower vertebrates information regarding structural and functional activities of ApoE is very limited. The annual fish (*Nothobranchius guentheri*) apolipoprotein E (NapoE) shares the same structural features as mammalian ApoE. High level of NapoE transcripts has been detected in liver, and its transcript level down regulates gradually with increase in age, making it as a suitable marker for aging, and supporting role of ApoE associates with longevity. Antioxidation property of recombinant NapoE was studied for both domains separately. It was found that the N-terminal domain inhibited oxidation of LDL contributing towards protection of lipid oxidation (Wang *et al*. 2014). The biological functions of most widely explored apolipoproteins, AI and E along with HDLs are also briefly explained (Fig. 2).

Apolipoprotein H

Apolipoprotein H, a beta-2 glycoprotein, directly scavenges lipopolysaccharide (LPS) layer present in outer membrane of Gram-negative bacteria. This is synthesized by liver cells and binds to monocytes as b2GPI-LPS complex by activating host immune system (Agar *et al*. 2011). Along with that, this protein helps in carriage and entry of hepatitis-B surface antigen (HBsAg) to hepatocytes. The presence of this protein has been reported in *L.rohita,* one of the Indian major carps as described in its immune-related EST analysis (Das *et al*. 2014).

Other apolipoproteins

ApoM is a lipocalin family of protein mainly associated with HDL (Karlsson *et al*. 2006).The N-terminal hydrophobic signal peptide binds to monolayer of lipids present in HDL (Xu *et al*. 2004). The secretion of this protein from liver depends upon ApoA-I contents in plasma. In human genome, ApoM is located in chromosome 6 on a position which is a highly conserved region for genes involved in innate immunity and inflammation,e.g. TNF (tumor necrosis factor), lymphotoxin A and B (Dahlbäck and Nielsen 2006). Hence, it may be possible that ApoM also plays some role in innate immunity. ApoMgene appears to be present in zebrafish, puffer fish and rohu (van Dijk*et al*. 2006, Mishra *et al*. 2011). The partial sequence information (530 bp) of rohu ApoM mRNA was generated using gene specific primers. The sequence showed the highest (82%) similarity with ApoM of *Hemibarbus mylodon,* an endangered Korean fish species. The expression of ApoM gene in rohu was found predominantly in liver tissues. However, heart and spleen tissues also

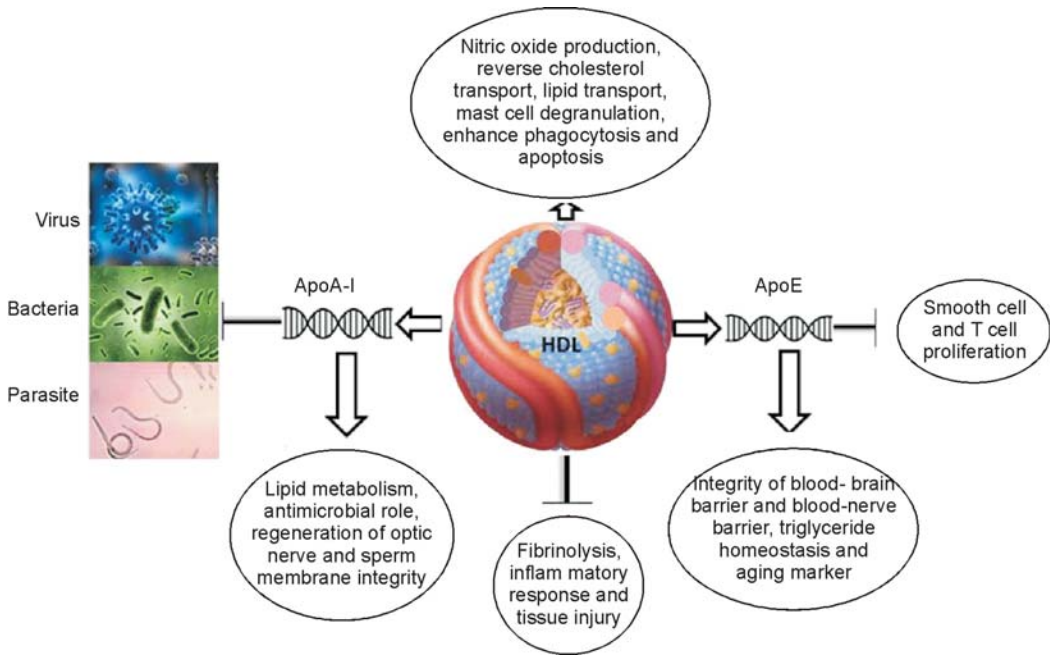

Fig. 2.Schematic representation of important biological properties of HDL, ApoA-I and ApoE.
(⊣ denotes inhibition)

showed faint expression and the expression was below detectable limit in all other tissues in rohu carp (Mishra *et al*. 2011).

Apolipoprotein J, which is otherwise known as clusterin, is a heterodimeric highly conserved secretory glycoprotein reported to be involved in diverse physiological processes such as stabilization of stressed proteins in a folding-competent state, promotion or inhibition of apoptosis, tissue remodeling, membrane recycling, cell-cell and cell-substratum interactions, sperm maturation, lipid transportation and complement inhibition. Up-regulation in the expression of ApoJ was observed during several neurodegenerative conditions, cancer progression and tumor formation (de Silva *et al*. 1990, Trougakos and Gonos 2002).

Except all these apolipoproteins, two new proteins ApoO and ApoL has also been reported in fish *Gobio cyprisrarus* with their partial sequence information. Till now, no specific functions of these proteins are being described in fish (Fang *et al*. 2009). However, ApoO helps in efflux of cholesterol from macrophage cells (Lamant*et al*. 2006).Human ApoL acts as an anti-parasitic agent towards *Trypanosoma brucei* infection and is regulated by multiple pro-inflammatory signaling molecules (Monajemi *et al*. 2002, Sana *et al*. 2005). The ApoL protein associated with HDL particles are being engulfed by *T. brucei* to fulfill their lipid and iron need. When it reaches lysosome, the alteration of pH from 7 to 5 induces conformational change in membrane addressing domain (MAD) of the protein, making release of ApoL from HDL into lysosomal membrane. Then ApoL creates anionic specific pores by activating its pore forming domain leading to lysis of pathogen (Smith and Malik 2009).

Phylogenetic relationship among multigene family apolipoproteins of one of the Indian major carps, rohu (L. rohita)

The carps are the most important aquaculture species in the world contributing 71.9% of total production in 2010. India being the second largest producer of all carps provides excellent scope for culture of rohu (*L.rohita*), catla (*Catla catla*) and mrigal (*Cirrhinus mrigala*), the Indian major carps. In India, total freshwater fish production reached 36,14,914 tonnes by 2010 and 94% of that were carps, and it produces about 70% of total Indian major carps. Annual production of 3 Indian major carps in Asia is 4,412,078 tonnes, of which rohu comprises 1,133,233 tonnes (FAO 2013). However, in spite of its consumer preference and susceptibility to various disease problems, very little is known about its immune system, particularly with regards to presence and functional roles of various apolipoproteins. Except a detailed study on rohu ApoA-I, no major work has been carried out in Indian carp species. To draw a preliminary idea about carps, the basic existence and sequence information on other apolipoproteins of rohuwere derived from EST data generated on this important species and the same was utilized to construct their phylogenetic diversity.

Phylogenetic analysis of *L. rohita* ApoA-I revealed that it formed the sister group with *Cirhinnus molitorella* and both species sharing a common ancestry with *Cyprinus carpio* and *Hypophthalmic hthysmolitrix*, appearing in the same clade. *L. rohita* ApoA-I also showed significant separation between the ApoA-I from teleosts and mammalian species, as indicated by their positions in different clusters (Mohapatra *et al*. 2016). Further, the individual partial sequence information available in EST database of *L. rohita* (Robinson *et al*. 2013) on various apolipoproteins was used to construct phylogeny tree. The sequence of individual apolipoprotein was aligned using Clustal X multiple alignment software and phylogeny was constructed using MEGA 6 software and tree was made by Neighbor joining method. Phylogenetic analysis of ApoB sequence of *L. rohita* forms sister group with human ApoB-100 (Fig. 3). However, zebrafish ApoBb formed a different sister group with *Sinocyclocheilus rhinocerous*. Phylogenetic tree of *L. rohita* ApoC-II clustered together with *S.rhinocerous* and shared a common ancestry with partial sequences of *Haplochromis mylodon*, *Danio rerio*, *Takifuguru bripes* and *Oncorhynchusmy kiss*. Mammalian species ApoC-II formed a different cluster showing significant separation between mammalian and fish species, except *S.rhinocerous* (Fig. 4).

Apolipoprotein A and innate immunity in fish

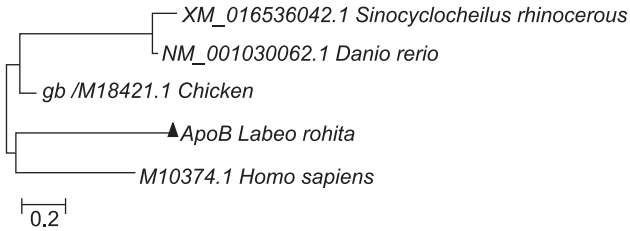

Fig. 3. Evolutionary relationship of taxa shown as a tree constructed by neighbor joining method based on the partial sequence alignment of ApoB. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogeny tree.

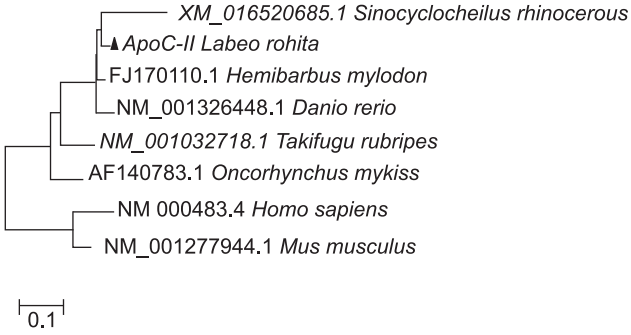

Fig. 4. Evolutionary relationship of taxa shown as an unrooted tree constructed based on the partial sequence alignment of ApoC-II. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogeny tree.

Table 2. Antimicrobial role of fish Apolipoprotein A-I

Fish species	Activity against pathogens studied	Biological function	Reference
Ictalurus punctatus	*Aeromonas hydrophila Micrococcus lysodeikticus*	Lytic activity and Immunostimulant	Pridgeon *et al*. (2013)
Gadus morhua L.	*Listonella (Vibrio) anguillarum*	Anti-bacterial activity	Caipang *et al*. (2008)
Oncorhynchus mykiss	*Escherichia coli*	Anti-bacterial activity	Villarroel *et al*. (2007, Dietrich *et al*. (2015)
Epinephelus coioides	*Vibrio alginolyticus,* Singapore grouper iridovirus (SGIV), *M. lysodeikticus* and *A. hydrophila*	Anti-viral and Anti-bacterial activity	Wei *et al*. (2015)
Epinephelus akaara	*Staphylococcus aureus, E. coli, Photobacterium damselae* and *Vibrio harveyi*	Anti-bacterial activity	Qu *et al*. (2014)
Morone saxatilis	*Streptococcus* sp., *E. coli* and *Mycobacterium marinum*	Anti-bacterial activity	Johnston *et al*. (2008)
Labeo rohita	*A. hydrophila* and *E. coli*	Anti-bacterial activity	Mohapatra *et al*. (2016)

AMPs are endogenous peptides having a broad spectrum of antimicrobial activity against various microorganisms. Most AMPs are amphipathic in nature, so they can directly act on the membrane of microorganisms forming a peptide-lipid interaction. By this interaction, peptides transport across the membrane producing a membranolytic effect (Hancock 1997, Hancock and Chapple 1999, Shai and Oren 2001, Rotem and Mor 2009, Huang*et al*. 2010, Nguyen *et al*. 2011, Teixeira *et al*. 2012). Apolipoproteins also possess the same structural features as general AMPs. They also have a high affinity towards the lipid that makes them effective antimicrobial agents against bacteria, viruses and parasites. Among all apolipoproteins, antimicrobial roles of ApoA-I and ApoA-II have been widely studied in many fish species. The major antimicrobial and immunomodulatory functions of ApoA-I against specific pathogens in various fish species were briefly described in Table 2.

Role during bacterial infections

Role of ApoA-I during bacterial infections has been widely studied from higher to lower vertebrates. This protein mainly provides protection against Gram-negative bacteria because of its high efficacy for binding to lipopolysaccharides (LPS) or endotoxin molecules present in bacteria. ApoA-I directly binds to LPS lipid A or it can bind through LPS binding protein indirectly (Ma*et al*. 2004). In channel catfish, the expression level of ApoA-I increased in all tissues (significantly in skin, anterior kidney and blood samples) except intestine after bath immersion with live *Aeromonas hydrophila* (Pridgeon *et al*. 2013). In *Epinephelus akaara* transcript level of ApoA-I increased in response to a Gram-negative bacteria *Photobacterium damselae* only in anterior kidney tissue and Atlantic codApoA-I expression significantly up-regulated in blood after vaccinated with heat-killed *Listonella anguillarum* (Caipang *et al*. 2008). Similarly, in *L. rohita* expression of *LrApoA-I* increased at 24 hpc (hour post-challenge) to 72 hpc with *A. hydrophila* and then declined to normal afterwards in anterior kidney tissue. But in liver tissue reduction in expression level was noticed during initial periods and a transient rise in ApoA-I expression was seen in 7 dpc (Mohapatra *et al*. 2016). Taken together this change in expression level of ApoA-I in different immunocompetent tissues of fish suggested that this protein plays an important role in defense during bacterial infections. Although a wide array of molecules of this class exists in fish, it needs to be looked into their functional role during bacterial pathogenesis.

Role during viral infection

Previously antiviral role of human ApoA-I has been evaluated against 3 viruses, xenotropic murine virus, human immunodeficiency virus (HIV) and herpes simplex virus (HSV). ApoA-I and its synthetic peptide inhibit viral penetration, cell to cell spread and viral induced cell fusion, neutralizing both DNA and RNA viruses (Kane*et al*. 1979, Srinivas *et al*. 1991, Alonso-Villaverde *et al*. 2003). In *Epinephelu scoioides* expression of ApoA-I was studied upon ploy I: C induction and challenged with Singapore grouper iridovirus (SGIV). In both the above cases, increased level of expression of ApoA-I gene was observed in liver tissue after 6 h of induction. Replication kinetics of the virus revealed that the growth of SGIV was also inhibited by ApoA-I gene and upregulation of other immune genes ISG15 and MX-I was also observed (Wei *et al*. 2015). Similarly, expression of *LrApoA-I* was evaluated in *L. rohita* upon induction with poly I: C in liver and kidney tissues. In liver high transcript levels were detected after 6 h up to 48 h of post- induction (hpi). And in kidney tissue a transient rise in expression was noticed at 24 hpi. All these data indicate potential antiviral role of ApoA-I from higher to lower vertebrates. However, the role of this molecule along with other classes of it needs to be investigated in detail in Indian fish species in terms of their antiviral effects by looking into any possible negative activity during virus

penetration, communication, multiplication or neutralization.

Role during parasitic infection

Role of apolipoproteins with regards to parasitic infection is very limited. The effect of ApoA-I was studied in aeukaryotic parasitic model (*Trypanosoma brucei*). ApoA-I helped in parasitic lysis by binding to trypanosome lytic factor (TLF), which is present in human serum (Gordon *et al*. 2011). In fish, role of ApoA-I gene during an ectoparasite infection, *Argulus*, was first reported by Mohapatra *et al.* (2016). *Argulus* is an ectoparasite that feeds on skin and mucus of the host releasing a toxin into the host. The estimated loss due to this parasite was recorded to be 29,524.40 INR/ha/year (Sahoo *et al*. 2013). Looking into the severity of disease, the expression of ApoA-I was studied in skin, mucus, kidney and liver tissues of *L. rohita*. Expression level revealed a million-fold increase in skin and mucus tissues on 15 days of post-infection, which was well correlated with degree of damage to the tissue during that period due to presence of adult parasites on skin. In liver and kidney tissues, the expression of ApoA-I showed also an increase at 3 days post-challenge with *Argulus*, thus indicating unique and significant role of this gene against parasitic infection. However, its potential role along with other molecules and their interaction with similar kind of molecules during any parasitic infection needs in depth investigation.

Antimicrobial and immunomodulatory roles of purified apolipoproteins

ApoA-I and ApoA-II were first detected in skin and mucus of carp (*Ciprinus carpio*). These HDL molecules were purified by affinity chromatography and exhibited bactericidal activity against non-pathogenic *Escherichia coli* (Beck *et al*. 2013). In another experiment, both ApoA-I and ApoA-II of carp were purified from HDLs by affinity chromatography and gel filtration, and its bactericidal and bacteriostatic activities studied against both Gram-positive (*Planococcus citreus*) and Gram-negative (*Pseudomonas* sp., *Yersinia ruckeri* and *E. coli*) bacteria. For *P.citreus* the EC_{50} (effective 50% reduction concentration) was found to be 0.4 μM and the concentration varied from 2.6–4 μM for Gram-negative bacteria (Concha *et al*. 2004). Similarly, antimicrobial role of rainbow trout ApoA-I tested against above same Gram-positive and Gram-negative bacteria. Its EC_{50} ranged from 0.3 to 0.8 μM for a wide range of bacteria except *E.coli* which was found to be 8 μM (Villarroel *et al*. 2007). Along with that plasma ApoA-I level was evaluated in healthy and sick fish, and band intensity of ApoA-I was more in sick fish than that of healthy fish (Villarroel *et al*. 2007). In Atlantic cod, ApoA-I expression was significantly up-regulated in blood after being vaccinated with heat-killed *Listonella anguillarum* along with other proinflammatory cytokines (Caipang *et al*. 2008). Antibacterial assay of recombinant ApoA-I from striped bass was evaluated against 3 bacteria, *E. coli*, *Streptococcus* sp. and *Mycobacterium marinum*. Significant reduction in growth was noticed at 125μg/mL for *E. coli*, 250μg/mL for *Streptococcus* sp. and 250 μg/mL for *M. marinum* and complete inhibition of growth were observed at 1,000 μg/mL for *E. coli*, 500μg/mL for *Streptococcus* sp. and 375 μg/mL for *M. marinum* (Johnston *et al*. 2008). The recombinant ApoA-I isolated from channel catfish contained the typical domain of apolipoproteins, and its lytic and antimicrobial activities were studied against *Micrococcus lysodeikticus* and *A. hydrophila*. It showed lytic activity when lyophilized *M. lysodeikticus* and *A. hydrophila* were used as substrates, lytic activity of the recombinant ApoA-I was 43,443 U/mg against *M. lysodeikticus* and the inhibitory concentration for *A. hydrophila* was found to be 6 μM and 12 μM (Pridgeon *et al*. 2013). Similarly, *Epinephelus akaara* ApoA-I (EaApoA-I) possessed antimicrobial activities against the Gram-positive bacterium *Staphylococcus aureus* as well as the Gram-negative bacteria *E. coli*, *Photobacterium damselae* and *Vibrio harveyi*, indicating that ApoA-I may have a broad-spectrum antibacterial property (Qu *et al*. 2014). Both ApoA-I and ApoA-II proteins were isolated and characterized in rainbow trout seminal plasma. These proteins showed antibacterial activity against *E. coli* suggesting its role in giving protection to sperm and reproductive tissue from microbial attack and maintain membrane integrity of sperm (Dietrich *et al*. 2015). Similarly, minimum bactericidal concentration (MBC) for recombinant ApoA-I protein of *L. rohita* was found to be 100 μg against *A. hydrophila* and *Edwardsiella tarda.* These studies remain as some of the examples for using this molecule as antibacterial agents and also opened up scope for looking into similar or better role, if any, played by its sister molecules. Looking into these AMPs perhaps would suggest alternatives to antibiotics which have not been seen any new discovery since last 3 decades.

Immunostimulant activity of ApoA-I was studied using *in vivo* experiments, and in channel catfish the intraperitoneal injection pcDNA-ApoA-I construct rendered 100% protection against *A. hydrophila* challenge at 48 h post stimulation (Pridgeon *et al*. 2013). Recombinant ApoA-I of *L. rohita* provided 55% protection against *A. hydrophila* at 12 h post-stimulation along with up-regulation of expression of proinflammatory cytokine (IL-1β) and lysozyme G levels. Thus,ApoA-I and ApoA-II both possesses a broad spectrum antimicrobial activities, and ApoA-I can serve as an effective immunostimulant against many fish pathogens rendering immediate protection to host.

Other roles of apolipoproteins

ApoA-I was also found to be associated with some neural diseases, like optic nerve regeneration in fish. In various species different apolipoproteins were used for the above biological functions.In mammal its ApoEthat helps in optic nerve regeneration, and in fish and birds ApoA-I does similar function. Upon injury to the optic nerve, some myelin degraded products inhibits the neural regeneration.

In carps ApoA-I might act as a scavenger to this product thereby forming a favorable condition for regeneration (Harel *et al*. 1989). In *Carassius auratusgibelio*, Apo-14 protein was found in liver and intestine in embryonic and larval stages of fish. Its role in organogenesis of digestive system in these stages was evaluated by knocking down the Apo-14 gene in endothelial cells of liver. Disruptions of digestive organs were observed after knocking down of this gene providing the evidence of digestive system organogenesis by Apo-14 gene (Xia *et al*. 2008). During embryonic stage of *Epinephelus coioides*, a protogynous hermaphroditic marine fish, Apo-14 played an important role in morphogenesis and growth of liver and brain tissues (Zhou *et al*. 2005).The involvement of fish Apo-14 in immune response is poorly described. The transcription of both Apo-14 and Apo-Ewas up-regulated in response to parasite infection in grass carp (Chang *et al*. 2005). Similarly, in *Nothobranchius guentheri* ApoE showed an age-dependent down-regulation and acts as a marker for aging (Wang *et al*. 2014). In rainbow trout elevated expression ApoA-I-1 and ApoA-I-2 was observed in aflatoxin B1-induced hepatocellular carcinoma (Delcuve *et al*. 1992). Similarly, up-regulation of Apo-14, ApoA-I and Apo-B was also noticed in fathead minnow when exposed to the toxicant 2, 4-DNT (Wintz*et al.* 2006). It is worthwhile to look into role of these class of molecules in pollution or toxin metabolism. Besides this, apolipoproteins affect food intake of fish, control neuron associated diseases, lipid metabolism and transport. Thus, the multifaceted roles of these proteins make them unique for their detail characterization in Indian fish species.

Apolipoproteins are main plasma proteins playing a diversified function, and however, their presence and roles are poorly understood in many fish species. Earlier these proteins were only known for lipid transport and metabolism. In the recent past, different immunological functions of these proteins were bought to the limelight. Among all the apolipoproteins only ApoA-I and ApoA-II antimicrobial activities were characterized in few fish species. All apolipoproteins share structural similarity which helps in binding to LPS and virus particles, thus exerting their antimicrobial role. As the different subspecies of this class of proteins have varied functions in higher vertebrates, it is essential to look into the presence and functional characterization of the individual apolipoproteins in different fish species. It is also essential to look into the mechanism of action and the modulation of downstream molecules during the induction process of these proteins. Role of ApoA-I during parasitic infection needs to be more focused based on the preliminary evidence of its increase in expression during ectoparasite infection in rohu. Besides, their roles during endo- or exotoxin metabolism or degradation along with tissue repair need to be understood. Strong co-relation between inflammatory and innate immunity response makes apolipoproteins a promising target for treatment or prevention of various diseases.

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