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Lrcasp9 shares similarity in structural motifs with human caspase-9 and is activated following bacterial infection and anti-viral vaccination

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Abstract

Among various caspases, caspase-9 plays a crucial role in the initiation phase of apoptotic cascade. To investigate about it in a high-valued freshwater fish species rohu (*Labeo rohita*), we cloned and characterized full-length caspase-9 cDNA (Lrcasp9) and analyzed its expression following bacterial infections and anti-viral vaccinations. The Lrcasp9 consisted of 1619-bp nucleotides (nt) having an ORF of 1302 nt encoding a polypeptide of 433 amino acids (aa) with a molecular mass of ~48.20 kDa. Structurally, Lrcasp9 comprised of one CARD domain (1–89 aa) and one CASc domain (161–430 aa). The CASc domain consisted of one large subunit (p20) spanning from 168 to 300 aa, and a small sub unit (p10) from 343 to 430 aa. The caspase family signature histidine active motif $H^{233}SAYDCCVVIILSHG^{247}$, cysteine active motif $K^{287}PKLFFIQACGG^{298}$ and pentapeptide "QACGG" active sites present in the p20 domain of Lrcasp9 was conserved across fish species, mouse and human caspase-9. Phylogenetically, it was closely related to common carp caspase-9 and exhibited significant similarity (90.1%) and identity (85.3%) in their amino acid sequence. In the uninfected fish, Lrcasp9 gene expression was highest (~5.3-fold) in blood and lowest in gill. In response to *Aeromonas hydrophila* and *Edwardsiella tarda* infection and rhabdoviral vaccination, Lrcasp9 gene expression was significantly ($p > 0.05$) enhanced in gill, liver, kidney and spleen, and also in vitro during cell death, suggesting activation of the intrinsic apoptotic pathway in bacterial infections and anti-viral vaccination in *Labeo rohita*.

Keywords Aeromonas hydrophila · Caspase-9 · Edwardsiella tarda · Labeo rohita

Introduction

Apoptosis (Kerr et al. 1972) is a regulated form of cell death that plays an essential role in multicellular organisms. The process is characterized by several biochemical features including cell shrinkage, membrane blabbing, chromosome condensation, nuclear fragmentation and the eventual engulfment of the cell by phagosomes. These biochemical changes are caused by the activation of a family of intracellular cysteine aspartyl-specific proteases known as caspases. Altogether, 15 caspases have been identified from mammals, of which 11 are from humans and other 4 are from mouse and cattle (Sakamaki et al. 2007). Mammalian caspases can be subdivided into three functional groups: initiator caspases (caspase-2, -8, -9 and -10), executioner caspases (caspase-3, -6 and -7), and inflammatory caspases (caspase-1, -4, -5 and -11 to -15). Initiator caspases initiate the apoptosis signal, while the executioner caspases carry out the mass proteolysis that leads to apoptosis. Inflammatory caspases do not function in apoptosis but are rather involved in inflammatory cytokine signaling and other types of cell death such as pyroptosis. Generally, caspases are synthesized as inactive zymogens and they share the same domain organization which contains 23–219 amino acid residues long N-terminal prodomain followed by a large subunit (p20 domain) and a small subunit (p10 domain) (Earnshaw et al. 1999). Initiator caspases usually have large prodomains containing protein–protein interaction domains but the effector caspases commonly have a short prodomain without any apparent protein motifs (Salvesen and Dixit 1997).

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Depending upon the apoptotic signal transduction, apoptosis occurs through two pathways; intrinsic and extrinsic pathway (Strasser et al. 2000). In the intrinsic apoptotic pathway, caspase-9 is the most important initiator caspase. It is also known as ICE-LAP6, MCH-6 or APAF (apoptotic protease activating factor)-3. It has a 130-aa prodomain mainly consisting of CARD domain, as well as a large and a small subunit. Like other members of the caspase family, caspase-9 is synthesized as an inactive zymogen and is activated through proteolytic processing. There are two main pathways for caspase-9 activation: within the apoptosome, a large protein complex, which consists of caspase-9, cytochrome c and APAF-1 (Saleh et al. 1999), or by proteolytic cleavage by a previously activated caspase, which involves its dimerization (Bitzer et al. 2002).

Whenever a cell gets stress, cytochrome c is released from mitochondria to the cytosol in response to the internal apoptotic signal. This molecule binds an adaptor protein APAF-1, which recruits initiator caspase-9 (via CARD–CARD interactions). Binding of APAF-1 and caspase-9 leads to the formation of a caspase-activating multiprotein complex called the apoptosome. When apoptosome is activated, initiator caspases such as caspase-9 will cleave and activate other executioner caspases. This leads to the degradation of cellular components for apoptosis.

In mammals, caspase-9 has been extensively investigated about its structure, regulation and the function but information on caspase-9 is limited in lower vertebrates (Krumschnabel and Podrabsky 2009). In sea bass, caspase-9 was cloned and characterized. In the untreated fish, the basal expression was low in all tested organs, but in bacterial infection, enhanced expression of caspase-9 was detected in the head kidney (Reis and Vale Ado 2007). In the large yellow croaker (*Pseudosciaena crocea*) and purse red common carp (*Cyprinus carpio*), caspase-9 was also cloned and its differential expression was also studied following various types of stimuli (Mu et al. 2010; Gao et al. 2013).

In the Indian subcontinent, rohu (*Labeo rohita*) is an economically important and widely cultivated fresh water fish species. To protect this fish species against various types of diseases, it is necessary to understand the molecular mechanism of pathogenesis regulated by apoptosis and various types of caspases. Considering these, we cloned, sequenced and characterized caspase-9 in rohu and studied its expression in various tissues following bacterial infections and viral vaccination.

Materials and methods

Ethics statement

All experimental protocols and methods involving live fish (*L. rohita*) in this study were carried out in accordance with the approved guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), Govt. of India, and were approved by the Ethics Committee of the Central Institute of Freshwater Aquaculture (CIFA), Indian Council of Agricultural Research (ICAR), Govt. of India.

Fish

Twenty healthy rohu (*L. rohita*) fingerlings free from any clinical symptoms of diseases (avg. wt 50 g each) were collected from the ICAR-Central Institute of Freshwater Aquaculture (CIFA), and were maintained in 500 L FRP tanks @ 50 fish/tank with continuous aeration facility. Five out of 20 fishes were kept as control group and other fishes were kept for the treatment group. Acclimatization was carried out before the experiment for 4 weeks and fishes were fed with commercially available carp diet once daily. During the experiment, the water temperature and pH were ~ 28 °C and ~ 7.5 respectively.

Bacteria

The response of caspase-9 gene expression in rohu was investigated using two different types of bacteria, viz., *Aeromonas hydrophila* (ATCC-35654) and *Edwardsiella tarda* (ATCC-15947). At first, both cultures were grown in LB medium at 30 °C for overnight, and were centrifuged at 5000 rpm for 5 min followed by two washings in phosphate buffered saline (PBS, pH 7.2). To each rohu fish (in the treated group of five fishes), 100 µl of PBS containing bacterial suspension (1×10^6 cells/fish) was injected intra-peritoneally (i.p) and was kept in aerated tank. After 24, 48 and 72 h of post-infection, samples, viz., gill, liver, kidney, spleen and blood were collected from both the control and treated fish and were put in TRIzol reagent for RNA extraction and further work.

Anti-viral vaccination

Besides bacterial challenges, to immunize fishes with viral antigen, an inactivated rabies vaccine Defensor1 (Zoetis, USA) was used. In the treated fish group, five fishes were injected with 100 µl of this vaccine intra-peritoneally (i.p) and the control fishes were injected with 100 µl PBS only. After 24, 48 and 72 h post-vaccination, gill, liver, kidney, spleen and blood were collected from both the control and treated fish and were put in TRIzol reagent for RNA extraction and further work.

RNA isolation and cDNA synthesis

Total RNA from tissue/cell culture samples was extracted with TRIzol reagent (Invitrogen, USA) following manufacturer's protocol. In each sample, the concentration of RNA was measured by UV-spectrophotometer (Eppendorf, Germany) and to examine the RNA quality, 1 µg of RNA from each sample was analyzed in 1% agarose gel. For cDNA synthesis, 1 µg of total cellular RNA was treated with 1 U of DNase I (MBI, Fermentas, USA) and reverse transcription was carried out using RevertAid 1st strand cDNA synthesis kit with oligo-dT primer (MBI, Fermentas, USA).

Cloning of rohu caspase-9

To clone rohu caspase-9 (Lrcasp9), total RNA was extracted from kidney and cDNA was prepared following the protocol as described previously. To amplify caspase-9 gene, PCR primers (Table 1A) were designed based on the nucleotide sequences of a small caspase-9 transciptome sequence obtained from rohu kidney by illumina sequencing and a second primer was designed from the conserved regions of common carp (GenBank: KC676314.1), zebrafish (GenBank ID: NM_001007404.2) and grass carp caspase-9 (GenBank ID: KT239368.1) cDNA sequence. In a 50 µl PCR reaction mixture, 1 µl rohu-kidney cDNA was used as a template and the PCR reaction was carried out with the following parameters: initial denaturation at 94 °C for 5 min followed

by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s and the final extension at 72 °C for 10 min. Then, 10 µl of the PCR amplified products were analyzed in 2% agarose gel. Then specific size of DNA band was purified from gel with QIAquick® Gel Extraction Kit (Qiagen, cat no-28704). Purified DNA was cloned into pGEM-T Easy vector (Promega, Madison, USA, Cat no. A1360) and isolated plasmids from recombinant white clones were DNA sequenced with T7 and SP6 primers at Bioserve, India Ltd, Hyderabad, India. The obtained sequences were analyzed by BLAST search (Altschul et al. 1990) at the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) to confirm them as rohu caspase-9. Further, a combination of the above primer sets was employed to conduct PCR. The specific band was purified from the gel, cloned in pGEM-T Easy vector and after both strand sequencing, the obtained sequences were joined with the previous sequence. To obtain full-length cDNA sequence of Lrcasp9, 5′ and 3′- rapid amplification of cDNA ends (RACE) were performed using the SMARTer RACE cDNA amplification kit (Clontech, USA). To amplify 3′-end, specific forward primer was designed from the 175-bp amplified sequence and for 5′-end, reverse primer was designed from a 145-bp partial Lrcasp9 gene sequence. Touchdown-PCR was employed for 3′ and 5′ RACE using GSP-FW-3′/UPM and GSP-RV-5′/UPM primer sets (Table 1A), respectively, under the conditions of one cycle of initial denaturation at 94 °C for 2 min; followed by 5 cycles of 94 °C/30 s, 65 °C/3 min

Table 1 Primers, their nucleotide sequence and application

Primer	Sequence (5′→3′)	Application
(A) Primers used for cloning of caspase 9 in *Labeo rohita*		
Casp9a-FW[#]	GGGGCCAAGACGTGACCAAGC	Cloning
Casp9a-RV[#]	TCGAGCACCATTTCCACACTGA	
Casp9b-FW*	GCCCAC**R**CCCAG**Y**GACATCCT	
Casp9b-RV*	TG**K**GAGAC**D**GCAT**Y**ATTTAC	
GSP-RV-5′	TCGAGCACCATTTCCACACTGA	5′ RACE
GSP-FW-3′	GCCCACACCCAGCGACATCCT	3′ RACE
UPM	Long: CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	RACE
	Short: CTAATACGACTCACTATAGGGC	
NUP	AAGCAGTGGTATCAACGCAGAGT	Nested RACE

Primer	Sequence (5′→3′)	GenBank Accession no.	T_m (°C)	Size (bp)	Application
(B) Primers used for gene expression analysis by quantitative real-time PCR (qRT-PCR) assay					
Casp9 FW	GCCCACACCCAGCGACATCCT	MG833834	55	175	Gene expression analysis by qRT-PCR
Casp9 RV	TTGGGAGACGGCATCATTTAC				
β-Actin FW	AGACCACCTTCAACTCCATCATG	EU184877.1	55	200	
β-Actin RV	TCCGATCCAGACAGAGTATTTACGC				

Primers marked with (#) were designed from a small transcriptome sequence of rohu caspase9, and (*) primers were designed from the conserved regions of caspase-9 cDNA sequence of common carp, zebrafish and grass carp. In the degenerate primer oligonucleotide sequence, "R"stands for A/G; "Y" for C/T; "K" for G/T and "D" for A/G/T

; next 5 cycles 94 °C/30 s, 60 °C/30 s, 72 °C/3 min; next 25 cycles 94 °C/30 s, 55 °C/30 s, 72 °C/3 min; and one cycle at 72 °C/5 min. Nested PCR for 5′ and 3′RACE was performed using Lrcasp9 specific GSP-FW-3′/NUP for 3′ and GSP-RV-5′/NUP for 5′ nested primer sets (Table 1A). For nested PCR, 1 µl of primary RACE-PCR product was used as a template with the following conditions: initial one cycle of 94 °C/2 min, then 30 cycle of 94 °C/30 s, 55 °C/30 s and 72 °C/90 s followed by one cycle of 72 °C/5 min. The PCR products were cloned in pGEM-T Easy vector, sequenced and validated through BLAST search (12).

Analysis of rohu caspase-9 cDNA sequences

The open reading frame (ORF) of rohu caspase-9 was predicted from the full-length cDNA sequence using ORF finder at web servers of the National Center of Biotechnology Information (http://www.ncbi.nlm.nih.gov/gorf). The amino acid sequences of Lrcasp9 and other fish and animal species were analyzed by SMART (http://smart.embl-heidelberg.de) program to identify various structural domains. To identify various motifs/domains in Lrcasp9, the amino acid sequences were analyzed in motif scan (https://myhits.isb-sib.ch/cgi-bin/motif_scan). Molecular weight and theoretical isoelectric point (pI) were predicted with compute pI/Mw tool (http://web.expasy.org/computepi/). The amino acid sequences of caspase-9 gene of other fish and animal species were obtained from the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/protein) and were aligned in Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) program. Phylogenetic tree of caspase-9 was constructed with the deduced amino acid sequences of caspase-9 of rohu and other animal species following neighbor-joining (NJ) method of MEGA4 program (Tamura et al. 2007).

Tissue-specific caspase-9 gene expression in rohu

To study the basal expression of caspase-9 gene, different tissues, i.e. gill, liver, kidney, spleen and blood, from two healthy rohu (avg. wt ~ 50 g) were collected using TRIzol reagent. From each sample, total cellular RNA was extracted and cDNA was prepared. By qRT-PCR assay, expression of caspase-9 and β-actin gene was analyzed and their mean value was graphically represented.

Real-time PCR analysis

The expression of target gene (caspase-9) and the reference gene (β-actin) were analyzed by quantitative real-time PCR (qRT-PCR) assay in Light Cycler®480II Real-time PCR detection system (Roche, Germany). The qRT-PCR reactions were carried out in duplicate wells of a 96-well plate and each well contained 10 µl of reaction volume with

following constituents: Template (cDNA) 1 µl, FW and RV primer (Table 1B) 0.25 µl each, 2× lightCycler®480 SYBR Green I master mix (Roche, Germany, Cat no. 04707516001) 5 µl and PCR grade H_2O 3.5 µl. The qRT-PCR was carried out by following set of parameters: 95 °C for 10 min (initial denaturation) followed by 45 cycles of 95 °C for 10 s (denaturation), 55 °C for 10 s (annealing) and 72 °C for 10 s (extension). For negative control, PCR was carried out without template (cDNA). Target specificity was determined by analysis of melting curve after each assay and the PCR products were analyzed in 2% agarose gel. Relative quantification of the target gene with the reference gene was calculated by $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001). The results obtained were expressed as mean ± standard error. The significant difference of gene expression between the control and treated fish groups was determined by the statistical software SPSS v16.0 with $p < 0.05$ as significance level.

A. hydrophila infection on LRG cell lines

To investigate the caspase-9 gene expression during cell death *L. rohita* gill (LRG), cell line (NRFC023) (Majeed et al. 2013) was infected with *A. hydrophila*. The LRG cell line was obtained from the National Repository of Fish cell line (NRFC) of the National Bureau of Fish Genetic Resources (NBFGR) Lucknow, India. The cells were cultured in L-15 medium containing 10% FBS and antibiotic (penicillin, 100 IU/ml; streptomycin, 100 µg/ml) solution and were seeded in six-well plates (BD Biosciences, Cat no. 353046) at 28 °C for 24 h. After 24 h, cells were washed with PBS and L-15 growth medium without antibiotic was added into each well. LRG cells were divided into control and treated groups keeping two wells as control and four wells as treated. Control group was kept untreated. For the treated groups, cells were infected with *A. hydrophila* (12×10^3 CFU) for 1 MOI (Multiplicity of Infection). After 30 min of incubation, the media was changed and the status of the cells (both control and treated) was studied under the microscope up to 3 h from the time of infection. At the designated time point, RNA was isolated from the control and treated cells and caspase-9 gene expression was studied by qRT-PCR assay.

Results

Cloning and characterization of rohu caspase-9 cDNA

The full-length cDNA of Lrcasp9 comprised of 1619 nucleotides (nt) with the following features: 172 nt 5′ UTR (untranslated region), 1302 nt ORF (open reading frame) and 145 nt 3′-UTR and was submitted in the GenBank with the

Acc.No. MG833834. The translation start (AUG) site positioned at 173–175 nt (nucleotide) and the deduced amino acids (aa) in Lrcasp9 consisted of 434 aa with the predicted isoelectric point (pI) of 5.62 and molecular mass of ~ 48.207 kDa. Prediction of protein domain in Lrcasp9 by SMART revealed one N-terminal CARD domain (1–89 aa) and one C-terminal CASc domain (161–430 aa) (Fig. 1).

Structural relationship of Lrcasp9 with other animal species

In Lrcasp9, the span and the position of the CARD and CASc domains shared high similarity with other fish species, mouse and human caspase-9 (Table 2). The deduced amino acid sequence of Lrcasp9 polypeptide shared highest (85.3%) identity with common carp casp9, followed by white cloud mountain minnow (80%), zebrafish (78.4%) and grass carp (75.7%), and lowest identity with the Indian catfish caspase-9 (66.9%). The amino acid sequence of CARD domain and CASc domain of Lrcasp9 was also compared with various animal species, and it showed highest identity and similarly with common carp caspase-9. Between the CASc and CARD domain of Lrcasp9, maximum identity and similarity were observed with CASc domain of various fish species and other animals. The CASc domain of Lrcasp9 showed highest identity (95.2%) and similarity (92.2%) with common carp, followed by white cloud mountain minnow, grass carp and zebrafish. The lowest identity (87.4) and similarity (78.5) were observed with the Indian catfish (Table 3).

In addition to CARD and CASc domain, various putative motifs in Lrcasp9 were also identified through motif scan search and it revealed a large subunit (p20) from 168 to 300 amino acid residue and the small subunit (p10) 343–430 aa within the CASc domain. The caspase family signature histidine active motif H^{233}SAYDCCVVIILSHG^{247}, cysteine active motif K^{287}PKLFFIQACGG^{298} and the caspase-9 pentapeptide "QACGG" active sites were located in the p20 domain (Fig. 2). Alignment of caspase-9 amino acid sequences of various fish species with higher vertebrates, it revealed that the human caspase-9 active site residues at Arg^{180}, His^{237}, Trp^{354}, Arg^{355}, as well as the cleavage site to generate p20 domain and p10 domain at Asp^{315} and Asp^{330} were also present in Lrcasp9.

Phylogenetic relationship of Lrcasp9

To identify the evolutionary relationship, a molecular phylogenetic tree was constructed based on pairwise alignments of Lrcasp9 amino acid sequence with various fish and animal species caspase-9. The result showed two clearly distinct clusters: one for fish (cluster-I), and the other one for higher vertebrates (cluster-II) (Fig. 3). Among fish species, rohu, common carp, white cloud mountain minnow, grass

carp and zebrafish caspase-9 formed a separate group, and within this group, Lrcasp9 was most closely related to the common carp forming a separate subgroup from other members. The yellow head catfish and the Indian catfish formed a separate group. The rainbow trout, croakers, sea bass and murrel caspase-9 also formed a separate group. The mouse and human caspase-9 formed a separate cluster, and was distantly related to rohu and other fish species caspase-9.

Tissue-specific expression of Lrcasp9

To investigate the tissue-specific expression of caspase-9, total RNA was extracted from gill, liver, kidney, spleen and blood, cDNA was prepared and samples were analyzed by qRT-PCR assay. The result revealed constitutive expression of caspase-9 gene in all examined tissues with varied intensity (Fig. 4). Among all examined tissues, caspase-9 expression was lowest in gill, and in comparison with gill (calibrator tissue) the highest expression was found in blood (~ 5.3-fold) followed by spleen (~ 4.8-fold), liver (~ 2.2-fold) and kidney (~ 1.1-fold).

Modulation of Lrcasp9 gene expression in *A. hydrophila* infection

To analyze the response of caspase-9 gene in bacterial infections, rohu fingerlings were infected with *A. hydrophila* and after the designated time course, caspase-9 gene transcripts were analyzed in gill, liver, kidney, spleen and blood by qRT-PCR assay (Fig. 5). Compared to the control, caspase-9 gene expression in the treated fish gill was ~ 35.3-fold at 24 h post-infection, and it reached to basal expression level at 48 and 72 h post-infection. In the liver, caspase-9 gene expression was highest (~ 2.3-fold) at 24 h and then it gradually decreased with the advancement of time. In the kidney, induction of caspase-9 at 24 h was noted as ~0.8-fold but it increased to ~ 3.8-fold at 48 h, and then it was decreased to ~ 1.8-fold at 72 h post-infection. In spleen, it was downregulated at all the time point. In the blood of infected fish, caspase-9 gene expression was marginally induced (~ 1.2-fold) only at 48 h but at other time points it was downregulated.

Modulation of caspase-9 gene expression in *E. tarda* infection

The *in vivo* response of caspase-9 gene was analyzed by infecting the rohu fingerlings with *E. tarda* and caspase-9 gene transcripts were analyzed in gill, liver, kidney, spleen and blood by qRT-PCR assay at three different time points (Fig. 6). Compared to the control, induction of caspase-9 gene in gill, kidney and spleen of the treated fish was highest (~ 17.4-, ~ 6.4- and ~ 3.1-fold, respectively) at 24 h post-infection. In the liver, caspase-9 gene expression was

(a)

5` UTR

acatggggaaggaagagaatcgaagcagcgagtgagcagttcagtcagttcagctcagttaaagctttgttgttttttaacgcgtttttgtatgttacttcc   100

Met Asp Pro Lys His Arg Lys
acaaataaattaacagtaattccgtcatattgaaaacgtctcatagccataaacattttttaatactgctaga **atg** gac cct aaa cac aga aag   193

Ile Leu Gln Leu His Arg Ser Asp Leu Val Ile Ser Leu Asn Leu Pro Asp Ile Tyr Asp Leu Leu Leu Ser Lys
ata ctt cag ctg cac agg tct gat cta gtc ata tca tta aat ctt ccg gac atc tac gat ttg ctt ttg tca aag   268

Gly Val Phe Thr Gln Asp Met Ile Asp Glu Ile Arg Ser Lys Gly Pro Arg Arg Asp Gln Ala Arg Gln Leu Val
ggg gtc ttc acc cag gac atg atc gat gaa ata cgg agc aag ggg cca aga cgt gac caa gcc agg caa ctg gtg   343

Thr Asp Leu Glu Thr Arg Gly Ser Gln Ala Phe Pro Ala Phe Leu Glu Cys Leu Arg Glu Thr Gly Gln His Asp
aca gac tta gag aca aga ggg agt cag gct ttc cca gcc ttc ctg gaa tgt ctg cgt gaa acc ggc cag cat gac   418

Leu Ala Glu Leu Leu Gln Cys Gly Asn Gly Ala Arg Leu Pro Pro Ser Val Pro Ile Gln Pro Ser Leu Ile Pro
ctg gca gag ctt ctt cag tgt gga aat ggt gct cga ctg cct cct tct gtc ccc ata cag cct tca ctg atc cct   493

Val Pro Val Glu Lys Pro Glu Leu Thr Glu Lys Pro Asp Arg Pro Asp Phe Thr Pro Pro Arg Leu Ala Pro Leu
gtg cca gtc gag aaa cct gaa tta aca gaa aaa cca gac aga cct gat ttt act cca cca cgg tta gca cca ctg   568

Arg Pro Glu Thr Leu Pro Val Pro Lys Pro Phe Ser Pro Ser Phe Glu Thr Gln Gly Val Arg Gln Arg Arg Asp
aga cca gaa acc ctg cct gtc ccc aaa cct ttt agc cca tct ttt gaa act caa gga gtg aga cag aga aga gac   643

Ser Ile Gln Cys Tyr Lys Met Asp Ala Ile Pro Cys Gly Ile Cys Leu Ile Ile Asn Asn Val Asp Phe Asp Pro
agt ata cag tgc tat aag atg gat gcc att ccg tgt ggg atc tgc ctg att ata aac aat gtc gat ttt gac cca   718

Gly Ser Glu Leu Lys Asp Arg Lys Gly Ser Asp Ile Asp Cys Asp Lys Met Glu Lys Arg Phe Lys Ala Leu Asn
ggc tct gaa cta aaa gat cga aag ggg tcc gac att gac tgc gac aag atg gag aaa aga ttt aaa gct cta aac   793

Phe Glu Val Ile Val Lys Arg Asn Leu Lys Tyr Lys His Ile Arg His Glu Met Ser Ser Leu Ala Lys Arg Asp
ttt gag gtt att gta aaa agg aac ttg aaa tac aaa cac ata aga cac gag atg tca tct ttg gca aag agg gat   868

His Ser Ala Tyr Asp Cys Cys Val Val Ile Ile Leu Ser His Gly Thr Glu Ala Asn His Asn Arg Phe Pro Gly
cat tct gca tat gac tgc tgt gtg gtg atc atc ctt tca cat ggt act gag gcg aac cac aat cgt ttt ccc gga   943

Ala Val His Gly Val Asp Gly Pro Ser Val Pro Val Gln Leu Ile Thr Asn Tyr Leu Asn Gly Gln Asn Cys Pro
gca gtt cat ggt gtt gac gga cca tct gtc cca gtt cag tta atc aca aac tac ctc aat ggc cag aac tgt cct   1018

Ser Leu Gln Gly Lys Pro Lys Leu Phe Phe Ile Gln Ala Cys Gly Gly Gly Glu Lys Asp Thr Gly Phe Glu Val
tct ctg cag ggc aag ccc aag ctg ttc ttc atc cag gcc tgt gga gga ggt gag aag gac acc ggt ttt gag gta   1093

Ser Ser Asp Glu Ala Glu Pro Ser Leu Gly Gly Ile Asp Asp Gln Thr Asp Ala Ile Pro Val Ser Ser Ser Ser
tct tca gat gag gct gag ccc tct ttg ggt ggg ata gat gac cag acg gac gct att cct gta tcc tcc agc agt   1168

Asp Ser Leu Ser Met Ser Asp Glu Pro Asp Ala Arg Ala Ser Leu Pro Thr Pro Ser Asp Ile Leu Val Ser Tyr
gac tct ctc agc atg tct gat gaa cct gat gct cgg gcc agt ttg ccc aca ccc agc gac atc ctg gta tcc tac   1243

Ser Thr Phe Pro Gly Tyr Val Ser Trp Arg Asp Thr Gln Ala Gly Ser Trp Tyr Val Glu Asn Leu Asp Arg Val
tca acc ttt cca ggc tat gtc tcc tgg agg gac acg caa gcc gga tcc tgg tat gta gag aat ctt gat cgt gtg   1318

Leu Glu Glu Asn Gly Ala Ile His Asp Leu Val Thr Met Leu Met Met Val Asn Asp Ala Val Ser Gln Ile Ser
ctt gag gag aat ggt gcc atc cat gat ctg gtg acc atg ctg atg atg gta aat gat gcc gtc tcc caa ata tct   1393

Ala Lys Gly Leu Tyr Lys Gln Met Pro Gly Ser Phe Asn Phe Leu Arg Lys His Leu Tyr Phe Gln Ser Ser Gln
gcc aaa ggc ctg tat aaa caa atg ccc gga tct ttc aac ttc ctc cga aaa cat ctc tac ttt cag tct tcg cag   1468

Ser   *
tca **tga** tcacttgaagatctttctttctttttctgctgtattttgaacactaccatgtacttgatatcatgtgataaaacctatttgttgttttttac   1566

atcataaactgagccatgtgatctaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa   1619

CARD (1-89 aa)

CASc (161-430 aa)

3` UTR

(b)

CARD   CASc

5`  [ 1-89 ]———————< 161-430 >  3`   433 aa

Fig. 1 Rohu caspase-9 full-length cDNA showing UTR, ORF, CARD and CASc domains. **a** Full-length caspase-9 cDNA sequence showing ORF (open reading frame), start and stop codons and un-translated regions (UTR) at 5′ and 3′-ends. Amino acids representing CARD domain (1–89 aa) has been shown in gray and the CASc domain in orange box. **b** Schematic representation of rohu caspase-9 with CARD and CASc domains as oval and hexagonal box at their respective positions

Table 2 Distribution of CARD and CASc domains in the caspase 9 of various fish and animals species

Species	Gen Bank accession no.	CARD aa	CASc aa
Labeo rohita	MG833834	1–89	161–430
Cyprinus carpio	AGM34043.1	1–89	164–433
Tanichthys albonubes	ACV31396.1	1–89	168–437
Ctenopharyngodon idella	ALB35228.1	1–50	125–394
Danio rerio	NP_001007405.2	1–89	163–433
Tachysurus fulvidraco	ARO86270.1	1–89	170–439
Clarias batrachus	AKE48162.1	1–95	168–435
Oncorhynchus mykiss	NP_001118119.1	1–89	162–432
Miichthys miiuy	AHG06616.1	1–89	164–433
Larimichthys crocea	XP_010753774.2	1–89	164–435
Dicentrarchus labrax	ABC70998.1	1–89	162–431
Channa striata	CCW28913.1	1–89	161–430
Homo sapiens	BAA82697.1	1–91	152–415
Mus musculus	NP_056548.2	1–91	190–453

The secondary structures of caspase-9 of various fish and animals were predicted by SMART program. The span and position of the caspase activation and recruitment domain (CARD) and the caspase interleukin-1 β-converting enzyme homologues (CASc) domain are shown

found to be ~ 0.8-, ~ 1.7- and ~ 1.2-fold at 24, 48 and 72 h of post-infection, respectively. In the blood, caspase-9 gene expression was downregulated with the advancement of time.

Modulation of caspase-9 gene expression against anti-viral vaccination

Following rhabdoviral vaccination, modulation of caspase-9 gene expression was investigated in different tissues (gill, liver, kidney, spleen and blood) of rohu fingerlings by qRT-PCR assay (Fig. 7). Compared to the control, induction of caspase-9 gene in the treated fish gill, liver and spleen was highest at 72 h post-infection, and it was ~ 5.8-, ~ 3.8- and ~ 3.2-fold, respectively. In the kidney and blood, caspase-9 gene expression was down regulated at each time point.

Lrcasp9 is induced during cell death following *A. hydrophila* infection

To investigate the caspase-9 gene expression during cell death, LRG cell line was infected with *A. hydrophila* followed by its microscopical observation and caspase-9 gene expression analysis by qRT-PCR assay (Fig. 8). The result showed that the uninfected control LRG cell lines maintained the morphology of the epithelial cells but *A. hydrophilla*-infected LRG cell line showed morphological changes of apoptosis, viz., shrinkage, clustering and detachment at 2 h. At 2.30 h, it was aggravated with most of the cells seen as floating and they were likely to undergo death. The qRT-PCR analysis of caspase-9 gene expression in the infected LRG cell lines revealed inductive expression of caspase-9 gene with the progression of time compared to the control. At 2 h post-infection Lrcasp9 gene expression was ~ 1.8-fold and it increased to ~ 2.3-fold at 2.30 h post-infection.

Table 3 Similarities and identity of Lrcasp-9 amino acids with other species (MatGAT analysis)

Species	Caspase9		CARD		CASc	
	Identity (%)	Similarity (%)	Identity (%)	Similarity (%)	Identity (%)	Similarity (%)
Cyprinus carpio	85.3	90.1	83.1	89.9	92.2	95.2
Tanichthys albonubes	80.0	87.3	73.0	86.5	91.5	94.1
Danio rerio	78.4	86.9	74.2	84.3	85.6	92.6
Ctenopharyngodon idella	75.7	81.5	45.6	49.4	90.7	93.7
Miichthys miiuy	71.9	81.6	70.8	83.1	82.2	89.6
Larimichthys crocea	70.9	81.0	69.7	83.1	80.5	88.6
Dicentrarchus labrax	69.9	79.1	65.2	80.9	81.1	87.8
Tachysurus fulvidraco	68.5	81.4	69.7	84.3	79.6	88.9
Oncorhynchus mykiss	67.4	79.4	65.2	80.9	80.8	90.0
Channa striata	68.3	80.1	68.5	80.9	79.3	88.1
Clarias batrachus	66.9	79.5	61.1	73.7	78.5	87.4
Homo sapiens	48.8	65.8	48.4	67.0	54.4	70.7
Mus musculus	48.1	64.3	53.8	71.4	54.1	69.6

```
L. rohita      MDPKHRKILQLHRSDLVISLNLPDIYDLLLSKGVFTQDMIDEIR------SKGPRRDQAR QLVTDLETRGSQAFPAFLECLRETGQHDLAELLQCGNGARLPPSVPIQPSLIPVFVEKP- 113
C.carpio       MDPRHRQILQRNRSNLVLTALNPKEICDGLLSKGVFTQDMIDEIR------SKIIRRDQAR QLVTDLETRGSQAFPAFLECLRETGQHALAELLQCGDGVRLPPSVPIQPSLIPLFVQNP- 113
T.albonubes    MDPKHRGILQRQRYNLVKALNPPEICDGLLSKGIFTQDVIDGIK------SKETRREQAR QLVTDLETRGSRAFPAFLECLCETGHQDIAELLQSEDGARHPLPIPVQPLQVPLFVQIP- 113
D.rerio        MEQKHRQILQRHRLNLVKALNPEDVCDKLLSKGVFTQDMIDEIQ------SKATRRDQAR QLVKDLETRGSLAFPAFLDSLRETGHNDLAELLQSGDGVHRPPVISVQPSPIPLFVQNL- 113
C.idella       ------------------MKYR------SKETKRDQAR QLVTDLETRGSQAFPAFLECLRETGHHDLVELLQSGDSAHRPPPIPVQPSLIPLFVQNP- 73
T.fulvidraco   MERRHKEILRRKRINLVLSLNPSDVYDGLLSRGIFTDDMIDEIK------NSGPRRAQAR QLVRDLETRGSLAFPAFLECLRETGQHTLAELLESGDPVPVPVPLPVQPSIIPLFVQPSI 114
M.miiuy        MEERHKKILQRNRTNLVRDLDPSKLYDGLLEKGVFTQDMIDEIK------SAGIRRDQAR QLVIDLETRGSRAFPLFLECLQETGQHSLAELLQNGAPPIQLQ-----PATPTQVVRPVL 109
D.labrax       MEDYRKRILQRNRTNLVRDLDPSKLYDGLEKGVFTQDMIDEIK------SSGTRREQAS QLVRDLETRGRRAFPLFVECLQETGQQLAELLQNEAPAVQLQ-----PATPTQVVRPVL 109
L.crocea       MEERHKKLLQRNRTNLVRDLDPSKLYDGLLEKGVFTQDMIDEIK------SAGIRRDQAR QLVIDLETRGSRAFPLFLECLQETGQHSLAELLQNGAPPIQLQ-----PATPTQVVRPVL 109
C.striata      MDEKHKNVLRRNRTNLVRDLNPSDLYDGLLEKGVFTQDMIDEIK------RSGTRRDQAR QLVRDLATRGSQAFPLFLECLKETGQQSLAELLENGAPGVQPQ--------PTAVVRPVV 106
O.mykiss       MEERHKRILQRNRVNLVSLDPSDMYDGLLSKGIFTQDMIDEIK------SAGIRRDQAR QLVRDLETRGSRAFPALIECLREKGQHSLPDLLLAGEPQSQPL-----P--LPVHVRPVV 107
C.batrachus    MDRRHKEILQKRRRELVSQLEPSDVYDGLLSRGILTDDMIDEIKVRALEKSSRPRRDQAR QLVKYLETRGSRAFTAFLECLTEQHTLADLLESGDSAPVSAPLPV-------PTQPTI 113
H.sapiens      MDEADRRLLRRCRLRLVEELQVDQLWDALLRELFRPHMIEDIQ----RAGSGSRRDQAR QLIIDLETRGSQALPLFISCLEDTGQDMLASFLRTNRQAAKLS----------------- 99
M.musculus     MDEADRQLLRRCRVRLVSELQVAELWDALLSRELFTRDMIEDIQ----QAGSGSRRDQAR QLVTDLETRGRQALPLFISCLEDTGGTLASLLQSGRQAAKQDPEAVKPLDHLVPVVL-- 114
                                                         :       :* ** **:  * ***  *:  ::..*  :.   :  .:*

L. rohita      ----------ELTE---KPDRPDFTPPR-----L--APLRPETLPVPKPF------SPS  FE-TQGVRQRRDSIQCYKMDAIPCGICLIINNVDFDPGSELKDRKGSDIDCDKMEKRFKA 205
C.carpio       ----------VYIE---KPDRPDFTPPL-----L--EPVRPKPLPVPNTPTPF---PGA  PV-VRPNRPRRDSIQCYKMDASPCGICLIINNVDFDPDCDLKDRKGSDIDCDKMEKRFKA 208
T.albonubes    ----------KYTE---KPDRPDLTPSV----VV--LPLKPEHLPLHKSLSPPDMKREN  QR-ERHKRPRRDSIQSYKMDASPCGTCLIINNVDFEPASDLKDRKGSNIDCDSMEKRFKA 212
D.rerio        ----------NHTE---KPDKPDIASPR-----L--VPLRPESLPVHKTYSPPF3----S  TV-VRPTRPRRDSIQCYKMDASPCGVCLIINNINFEKASELNDRKGSNIDCDKLEKRFKA 205
C.idella       ----------KCTK---KPDRPDLTPPV-----AV--VPLRPVSLPVVKPPSPP---SEA  QR-VRHDRLRRDSIQCYKMDASPCGICLIINNVDFEPASELKDRKGSNIDCDKMENRFKA 169
T.fulvidraco   IPLPVSGN--LLDFK---KPEGGDAP--K----KP--IPLKPKTLPIASI------EDET  EI-PRSTRPRSDSIQSYKMEASPCGVCLIINNVEFDSGSELKNRLGSDIDCEKLERRFRS 214
M.miiuy        QPIPLSSP--MDVDK--R-IKD----------E--VPLYPVPRPTTTPSPSPE--REY  IRPRPQGRPRRDSIQCYKMDASPCGHCLIINNVEFEPRSELSNRKGSNIDCDKLESRFKA 208
D.labrax       HPLPVSSP--MDVDK--R-KKD----------E--VPVYPKQIHSTTPTPSPE--REY  MR--PQGRTRRDSIQTYKMDCSPCGHCLIINNVEFAPQSELSNRKGSNIDCDKLRRFKA 206
L.crocea       QPIPVSSP--MDVDK--R-IKD----------E--VPLYPVPRPTTTPSPSPE--REH  IRPRPQGRPRRDSIQCYKMDASPCGHCLIINNVEFESRSDLSNRKGSNIDCDKLESRFKA 208
C.striata      RPLPVSPP--TDVNE---E-KKD----------D--APYDFKQTRNITPTTPSPE--MEY  MRPNQQGRIRRDSVQNYKMDASPCGHCLIINNVEFDPKSYLSNRRGSNIDCDKLEQRFKA 205
O.mykiss       RPLPVYSH--VDTVV---TPVYP----------S--QPVRPLP-TPTTPSLSPE--REF  GKPRATGRVRRDSIQSYKMDASPCGHCLIINNVEFEPQSELNNRTGSNIDCDKLERRFQS 206
C.batrachus    IPLPVR-K--PVDFK---KPERDDIP--E----DK--HLVKPTTLLIAPT------QNGT  EM-PRPPRPRSEISIPFYKMEANPCGICLIINNVEFDTNSVLKNRKGSDIDSEKLERRFRA 212
H.sapiens      ------------------KPTLENLTPVVLRP-----EIRKPEVLRPETPRPVDIGSGGF  GDVGALESLRGNADLAYILSMEPCGHCLIINNVNFCRESGLRTRTGSNIDCEKLRRRFSS 196
M.musculus     GPMGLTAKEQRVVKLDPSQPAVGNLTPVVLGPEELWPARLKPEVLRPETPRPVDIGSGGA  HDVCVPGKIRGHADMAYTLDSDPCGHCLIINNVFCPSSGLGTRTGSNLDRDKLEHRFRW 234
                                                                         *  .:  .***  ****** :: *     .  *  * **::*  :.:.  **

                                histidine active site                                cysteine active site
L. rohita      LNFEVIVKRNLKYKHIRHEMSSLAKRIHSAYDCCVVIILSHGTEANHNRFPGAVHGVDGP  SVPVQLITNYLNGQNCPSLQGKPKLFFIQACGGEKDTGFEVSSDEAEPSLGGI--DDQT 323
C.carpio       LNFEVIAKRNLKHHKRIRHEMTSLAKRIHSAYDCCVVIILSHGTEANHNRFPGAVHGVDGP  AVPVQLITNYLNGQNCPSLQGKPKLFFIQACGGEKDTGFEVSPDEVEPSLGGI--DDQM 326
T.albonubes    ALPVQLITNYLNGKNCPSLQGKPKLFFIQACGGGEKDIGFEVSPDEVEPSSGGI--DDQM 330   [LNFEVIVKRNLKYKHIRHEMSSLAKRIHSEYDCCVVIILSHGTEANHNRFPGAVHGVDGP]
D.rerio        LNFEVTVKRNLKSKRIRHEMASLAKKIHSTYDCCVVIILSHGTEASHNRFPGAVHGVDGP  AVPIQIITNYLNGQNCPSLQGKPKLFFIQACGGGEKDIGFEVSPDDVQPSIGGI--DDEM 325
C.idella       LNFEVIVKRNLKHIHIKRHAMSSLAKRIHSAYDCCVVIILSHGTEVNHNRFPGAVHGVDGQ  VVPVQLITNYLNGQNCPSLQGKPKLFFIQACGGEKDTGFEVSPDEVEPSLGGI--DDQM 287
T.fulvidraco   LNFHVLVKRNLKCKHIRHELATLAKMIHSVYDCCVVIILSHGTEASHNRFPGAVHGVDGL  SVAVQIITNYLNGQNCPSLQGKPKVFFIQACGGETDTGFEVSPDEVQHTAPRL--DEQA 332
M.miiuy        LNFIVEVKTNLKQKQIKHELSALCKMIHSQYDCCVVIMLSHGTEVSHSRFPGAVYGVDGL  HVPVQNITNYLNGKNCPSLQGKPKLFFIQACGGGEKDTGFEVSPDEVQPSIGGA--DDQT 326
D.labrax       LNFIVEVKTNLKQKQIKHELSALSKKIHSQYDCCVVIMLSHGTEVSHNRFPGAVFGVDGQ  YVQVQHITNYLNGQHCPSLQGKPKLFFIQACGGGDEKDHGFEVSPDEVEPSIGGA--DDQT 324
L.crocea       LNFIVEVKTNLKQRQIKHELSALCKMIHSQYDCCVVIMLSHGTEVSHSRFPGAVYGVDGL  HVPVQNITNYLNGKNCPSLQGKPKLFFIQACGGGEKDTGFEVSPDEVQPSIGGADADDQT 328
C.striata      LNFIVEVKLNLTQKQIKHALHTLSKKIHSQYDCCVVIILSHGTEVSHSRFPGAVYGVDGQ  CISVQNITNYLNGQNCPSLQSKPKLFFIQACGGGEKDTGFEVSSDEAEPSPGGA--DDQT 323
O.mykiss       LNFIVLVKRNLKQKQIRQELSALSKKIHSQCDCCVVIMLSHGTEVSHNRFPGAVHGVDGP  NVPVQLITNYLNGQNCPSLQGKPKLFFIQACGGGERDTGFEVTPDEDRPCIGGT--DDQT 324
C.batrachus    LNFEVIVKRNAKCKHIKHELSSLAKKIHSAYDCCVVVILSHGTEANHNRFPGAVHGVDGL  SVPVQIITNYLNGQNCPSLQGKPKLFFIQACGGNMRDTGFEVSSE--EHTAVRL--DEQV 328
H.sapiens      LHFMVEVKGDLTAKKMVLALLELAQQDHGALDCCVVVILSHGCQASHLQFPGAVYGTDGC  PVSVEKIVNFNGTSCPSLGGKPKLFFIQACGGGEQKDHGFEVASTSPEDESPGS--NPEP 314
M.musculus     LRFMVEVKNDLTAKKMVTALMEMAHRNHRALDCFVVVILSHGCQASHLQFPGAVYGTDGC  SVSIEKIVNIFNGSGCPSLGGKPKLFFIQACGGGEQKDHGFEVACTSSQGRTLDS--DSEP 352
                *.* *.* .:  .*       **  ** **:****** :::.* .:*****.*.**    .  .:  .  * .* ****  .***:********   * ****:  .    ::

                A-X-P-X                              small subunit (p10)
L. rohita      IAIPVSSSSDSLSM-SDEPDARASLPTPSDILVSYSTFPGYVSWRDTQAGSWYVENLDRV  LEENGAIHDLVTMLMMVNDAVSQISAKGLYKQMPGSFNFLRKHLYFQSSQS------ 433
C.carpio       IAIPISSSSDSLST-SDELDARATLPTPSDILVSYSTFPGYVSWRDTQAGSWYVENLDRV  LEENAATDDLVTMLMMVNNAVSQISAKGLYKQMPGSFNFLRKHLYFQASLS------ 436
T.albonubes    IAIPMSSSSDSLST-SDEPDARASLPTPSDILVSYSTFPGYVSWRDTQAGSWYVENLDRV  LEENAATDDLVTMLMMVNDAVSQISAKGLYKQMPGSFNFLRKLLYFQSPQP------ 440
D.rerio        IAIPMSSSSDSLSTASDELDARASLPTPSDILVSYSTFPGYVSWRDTEAGSWYVENLDRV  LEENAITDDLVTMLMLVNDAVSQISAKGLYKQMPGSFNFLRKLLYFQSSQS------ 436
C.idella       IAIPMSSSSDSLST-SDELDARATLPTPSDILVSYSTFPGYVSWRDTEAGSWYVENLDRV  LKENVATDDLVTMLMMVNAAVSQISAKGLYKQMPGSFNFLRKHLYFQSL-------- 395
T.fulvidraco   IAIPTSSSSDSLSL-SDEPDARATLPTPSDILVSYSTFPGYVSWRDTNKGSWYVETLDRV  LENNADTDDLVTMLMMVNNEVSQISAKGLYKQMPGSFNFLRKLFYFQVLKT------ 442
M.miiuy        IAIPMSSSSDSLSM-SDEPDARATLPTPSDILVSYSTFPGYVSWRDTQAGSWYEILDRI  LEENAATDDLGTMLMMVHEVSQNSAKGIYKQMPGSFNFLRKLLYFQSQA------- 435
D.labrax       IAIPTSSSSDSLST-SDELDARASLPTPSDILVSYSTFPGYVSWRDTQSGSWYVEILDRI  LEENAVTHDLVTMLMMVNEVSQNSAKGLYKQMPGSFNFLRKLLYFQSQSSS----- 435
L.crocea       IAIPMSSSSDSLSM-SDEPDARATLPTPSDILVSYSTFPGYVSWRDTQAGSWYVEILDRI  LEENAATDDLGTMLMMVNHEVSQNSAKGIYKQMPGSFNFLRKLLYFQSQA------- 437
C.striata      IAIPMSSSSDSLSM-SDEVDAKASLPTPSDILVSYSTFPGYVSWRDPQSGSWYVETLDRI  LEENAAVHDLATMLTMVNNDVSQNSAKGIYKQMPGSFNFLRKFLYFQTKA------- 432
O.mykiss       IAMPMSSSSDSLSTPSDEPDARATLPTPSDILVSYSTFPGYVSWRDTQAGSWYVETLDNV  LEDNAANDLVTMLMMVNHEVSQNSAKGLYKQMPGSFNFLRKLLYFQVLPCQRPIQA 441
C.batrachus    IAIPTSSSSDSLSL-SDEPDARATLPTPCDMLVSYSTFPGYVSWRDTNKGSWYVETLDRI  LEYNATTDDLATMLIMVNNEVSQISANGLYKQMPGSFNFLRKLFFFQVPKT------ 438
H.sapiens      IATPFQEGLR----TFDQLDAISSLPTPSDIFVSYSTFPGFVSWRDPKSGSWYVETLDDI  FEQWAHSEDLQSLLLRVANAV---SVKGIYKQMPGCFNFLRKKLFFKTS-------- 416
M.musculus     IAVPYQEGPR----PLDQLDAVSSLPTPSDILVSYSTFPGFVSWRDKSGSWYIETLDGI  LEQWARSEDLQSLLLRVANAV---SAKGTYKQIPGCFNFLRKKLFFKTS-------- 454
                ** * ...     *: ** ::****.*::*******:***** ; ****:* ** ::  .** ::*  *   *.:* ***:**.****** ::*:
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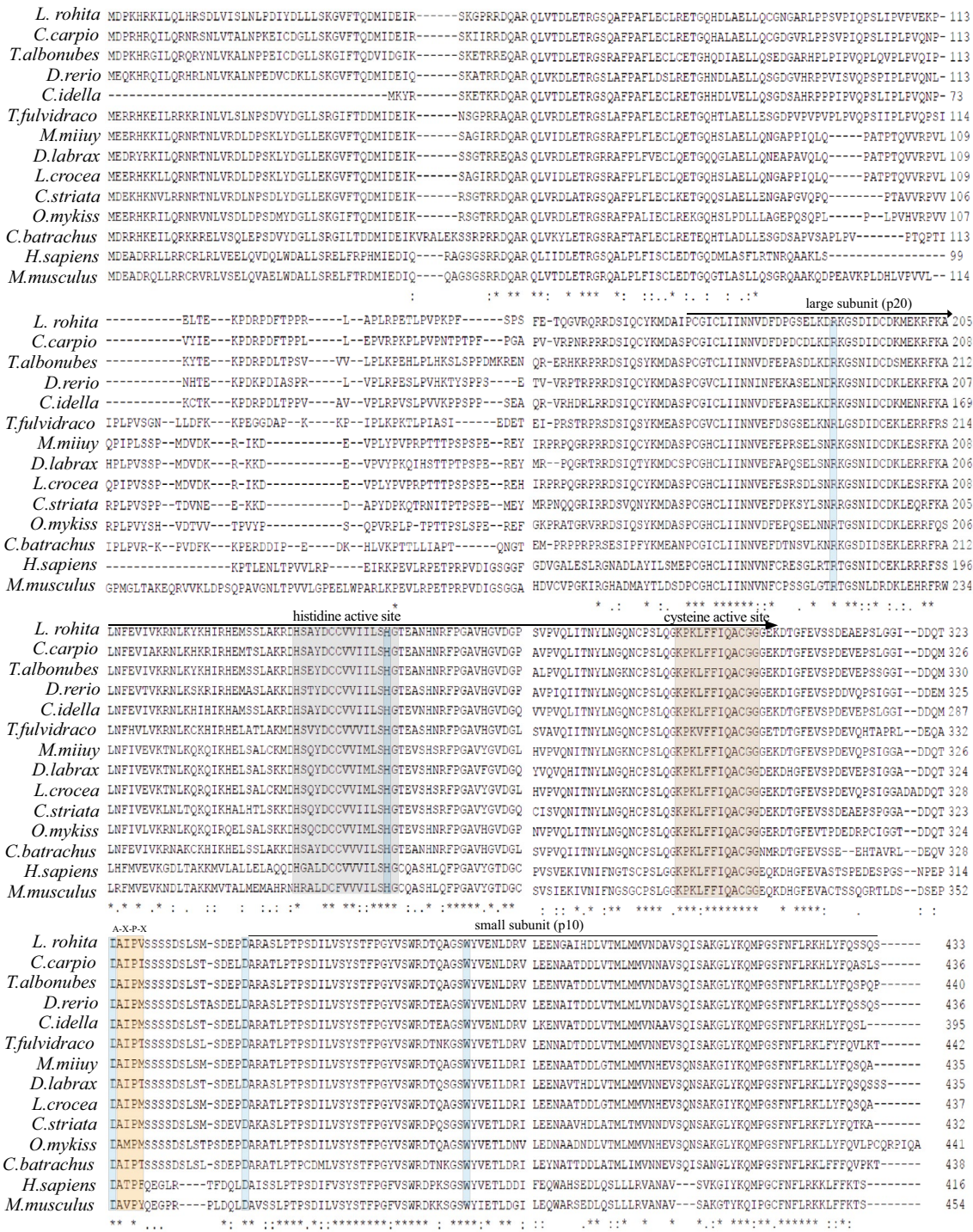
Fig. 2 Structural motifs present in Lrcasp9 and other fish and animal species. The amino acid sequences were retrieved from the GenBank data base and were aligned through Clustal Omega. The "dash" indicates gaps introduced to optimize similarity among sequences, "asterisks" denotes fully conserved amino acid residues in all sequences, "colon" represents conservation between groups of strongly similar properties and "dot" conservation between groups of weakly similar properties. The large (p20) and small (p10) subunits are shown by arrow and are marked. The histidine and cysteine active sites and the A-X-P-X motifs in the caspase family are marked. The caspase-9 active sites residues at Arg, His, Trp and the site of cleavage site for large and small subunit at Asp acid residue are marked with cyan box

Fig. 3 Phylogenetic relationship of rohu caspase-9 with other fish animal species caspase-9. Full-length caspase-9 amino acid sequences of various animal species were retrieved from the GenBank (Acc. no. mentioned inside the bracket), and the un-rooted phylogenetic tree was generated by the neighbor-joining method within the MEGA4 program
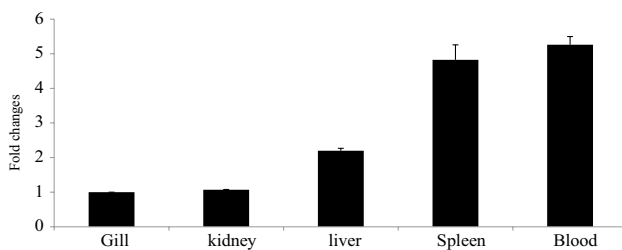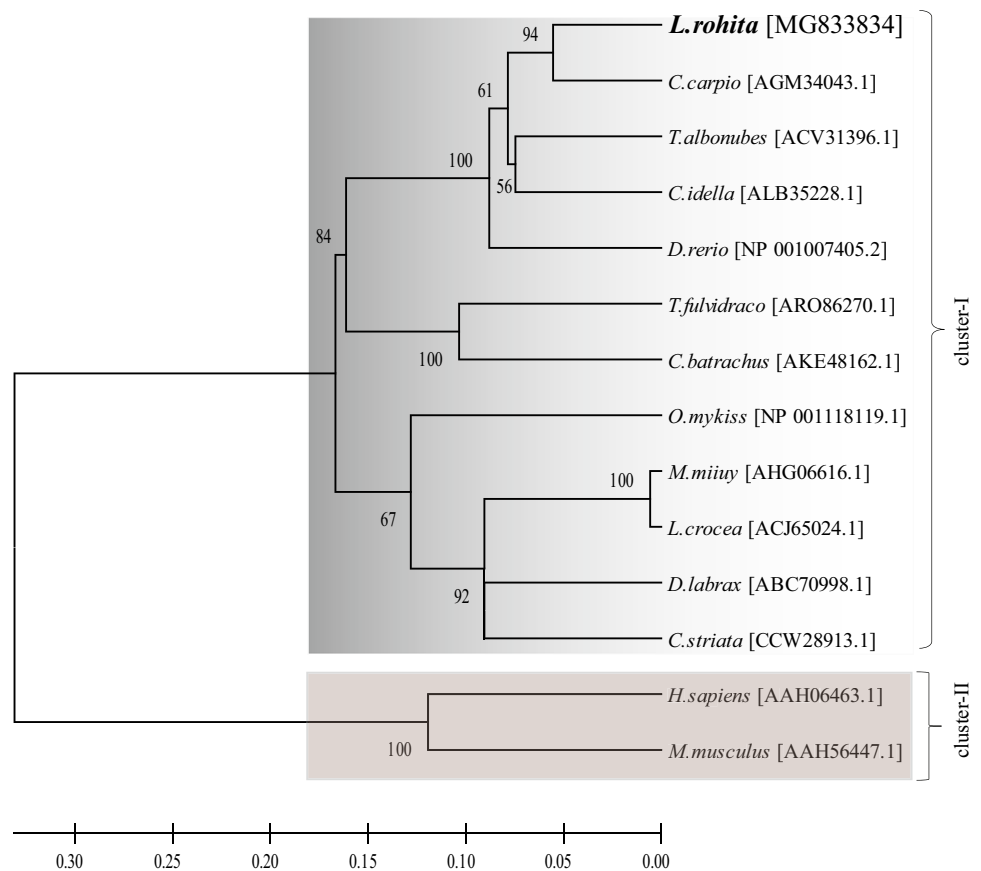

Fig. 4 Tissue-specific expression of caspase-9 gene in rohu. Total RNA was extracted from gill, liver, kidney, spleen and blood, cDNA was prepared and samples were analyzed by quantitative real-time PCR (qRT-PCR) assay. Expression of Lrcasp9 gene transcript in each tissue was represented as a ratio relative to β-actin (internal control) levels in the same samples. Gill was chosen as calibrator (1) and the relative expression of Lrcasp9 in other tissues was represented as fold changes from the calibrator. The results obtained from five fish ($n = 5$) were expressed as mean ± standard error (bars in the graph)

Discussion

Apoptosis is a complex processes involving a cascade mechanism that employs many proteins. However, the key enzymes in this process are the caspases, a family of cysteine proteases that control and mediate the apoptotic response. In comparison with higher vertebrates, information on caspases in various fish species is not elaborate. In rohu, not many studies on caspases have been carried out in spite of its importance as the leading freshwater fish species in the Indian subcontinent. Therefore, this study was undertaken to clone and characterize rohu caspase-9.

Analysis of *L. rohita* (rohu) caspase-9 (Lrcasp9) cDNA sequence in BLAST (Altschul et al. 1990) search revealed its homology with other fish and animal species caspase-9. Prosite analysis and motif search of the deduced amino acid sequence of Lrcasp9 showed typical caspase-9 architecture comprising CARD domain at the N-terminal and CASc at the C-terminal end. Both p20 and p10 subunits were also located in the CASc domain as observed in sea bass (Reis and Vale Ado 2007) and large yellow croaker (Mu et al. 2010). To identify the Lrcasp9 homologs, amino acid sequences of caspase-9 of fish and non-fish vertebrates were retrieved from the GenBank, and alignment of these sequences revealed high similarity particularly in the catalytic domain. It indicates that the caspase-9 is evolutionarily conserved from fish to higher vertebrates and may share the functional similarity across the species. The distinctive pentapeptide "QACGG" active site of caspase-9 (Reis and Vale Ado 2007; Duan et al. 1996) was also conserved in the Lrcasp9 and was located inside cysteine active site of
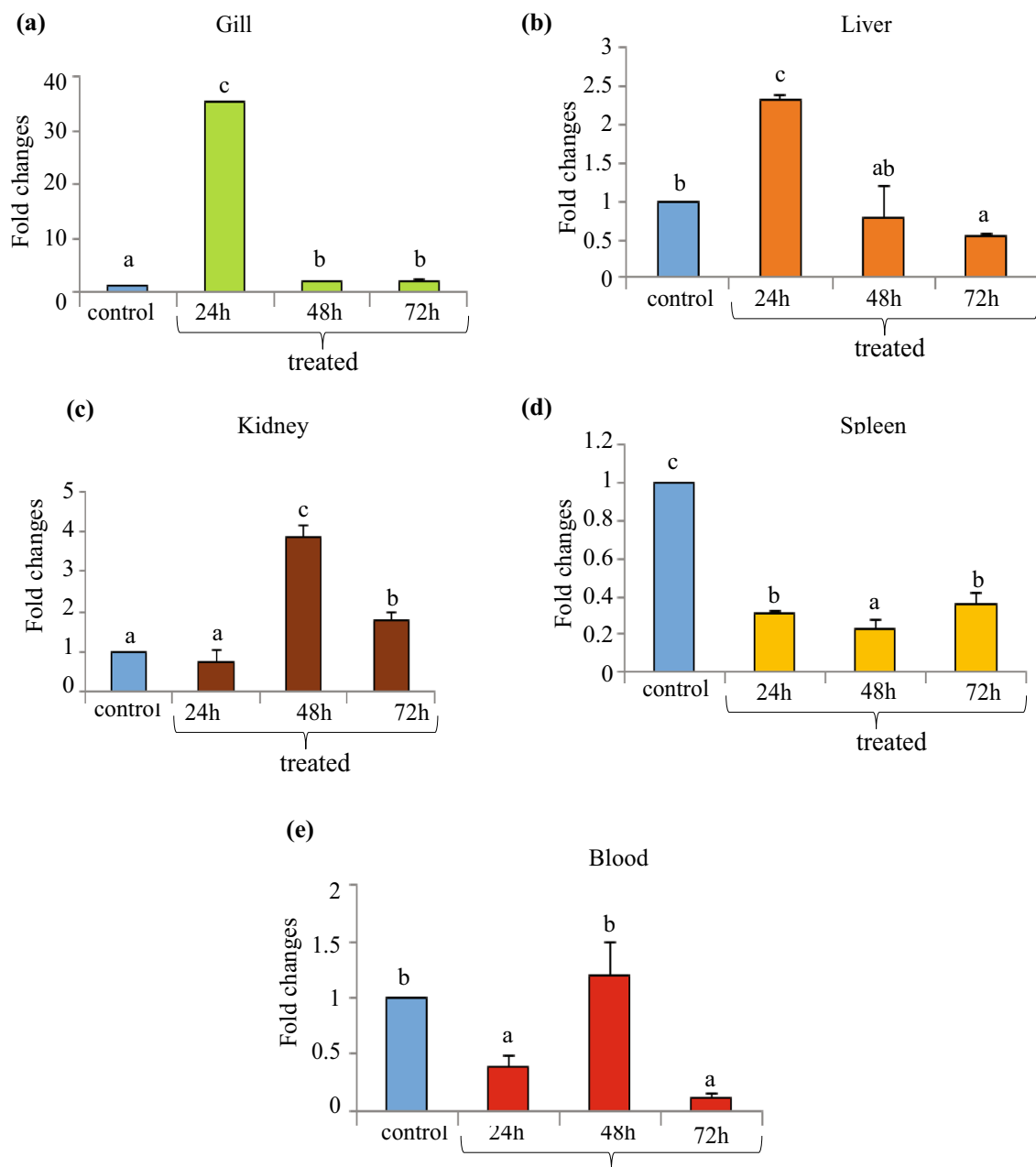
Fig. 5 Modulation of Lrcasp9 gene expression in *A. hydrophila* infection. *A. hydrophila* (1×10^6 CFU/fish) was injected into rohu fingerlings by intra-peritoneal (i.p.) route. After the designated time course, total RNA was extracted from gill, liver, kidney, spleen and blood. Quantitative real-time PCR was conducted to analyze caspase-9 and β-actin gene expression in various tissues. Data were normalized with β-actin and expressed as fold changes relative to the control. The results were expressed as mean ± standard errors (bars). **a** Gill, **b** liver, **c** kidney, **d** spleen, **e** blood

the P20 domain. The cleavage site between large and small subunit of human caspase-9 was placed at $PEPD_{315}$ and $DQLD_{330}$, and were reported as granzyme B and caspase-3 cleavage sites (Srinivasula et al. 1996). The first cleavage site of human caspase-9 at Asp ^{315} generates N-terminal ATPF (Ala–Thr–Pro–Phe) motif that can interact with the inhibitor of apoptosis protein (XIAP) to inhibit the caspase processing activity of the apoptosome (Srinivasula et al.

2001). Interestingly, Lrcasp9 and all other fish species caspase-9 also possessed "A–X–P–X" tetrapeptide motif and was similar to the "ATPF" motif of human caspase-9 and "AVPY" motif of the mouse caspase-9 (Fig. 2). These findings possibly indicate that the fish caspase-9 will have same cleavage site as observed in human. Multiple alignment and homology comparison revealed that, similar to human caspase-9, this potential cleavage site was also present in
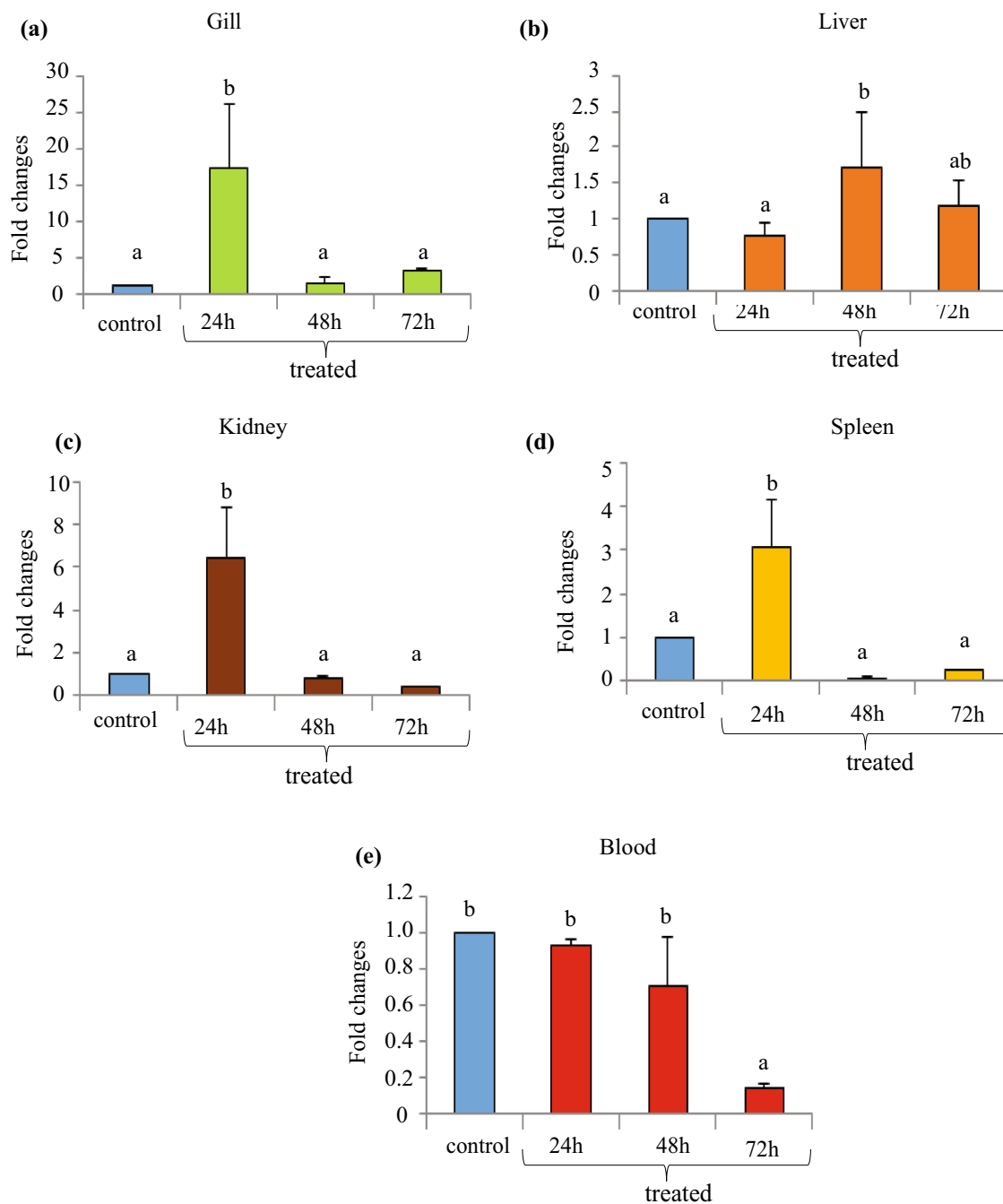
Fig. 6 Modulation of Lrcasp 9 gene expression in *E. tarda* infection. *E. tarda* (1×10^6 CFU/fish) was injected into rohu fingerlings by intra-peritoneal (i.p.) route. After the designated time course, total RNA was extracted from gill, liver, kidney, spleen and blood. Quantitative real-time PCR was conducted to analyze caspase-9 and β-actin gene expression in various tissues. Data were normalized with β-actin and expressed as fold changes compared to the control. The results were expressed as mean ± standard errors (bars). **a** Gill, **b** liver, **c** kidney, **d** spleen, **e** blood

Lrcasp9 at $DQTD_{324}$ and $DEPD_{342}$ and was well conserved in all fish and non-fish species. The putative cleavage site that separates the prodomain from the p20 domain in human at Asp_{130} (Duan et al. 1996) was not well marked in Lrcasp9 and other fish species caspase-9. This may be due to the high variability of prodomain among fish species. Most of the fish species, human and mouse caspase-9 contained a CARD domain of ~ 90 aa residues, histidine and cysteine active sites and characteristic-conserved amino acid residues at specific positions in human casp9 (Renatus et al. 2001). In Lrcasp9, we detected a similar span of CARD domain, histidine and cysteine active sites and conserved amino acids (Fig. 2).

مدينة الملك عبدالعزيز
KACST للعلوم والتقنية
🖄 Springer

(a)

Gill

(b)

Liver

(c) Kidney

(d)

Spleen

(e)

Blood
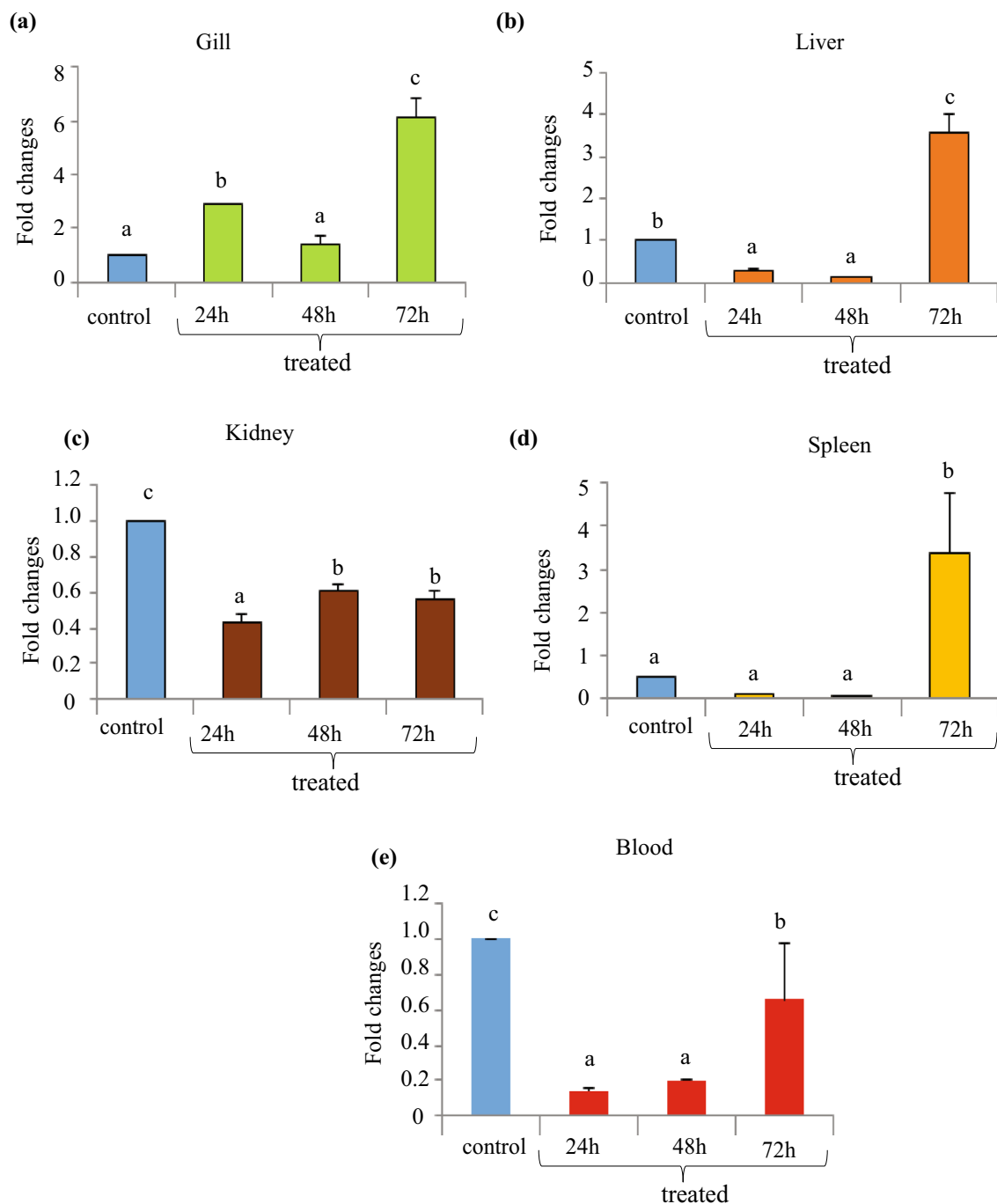
Fig. 7 Modulation of Lrcasp9 gene expression in inactivated rhabdoviral vaccination. Inactivated rhabdovirus vaccine was injected into rohu fingerlings by intra-peritoneal (i.p.) route. After the designated time course, total RNA was extracted from gill, liver, kidney, spleen and blood. Quantitative real-time PCR was conducted to analyze caspase-9 and β-actin gene expression in various tissues. Data were normalized with β-actin and expressed as fold changes relative to the control. The results were expressed as mean ± standard errors (bars). **a** Gill, **b** liver, **c** kidney, **d** spleen, **e** blood

All these features together suggest evolutionary conserved function of caspase-9 from lower aquatic animal like fish to human.

Phylogenetic analysis revealed that caspase-9 of fish species formed a separate group and was much apart from the higher vertebrates. This was expected, as high degree of similarity and identity among the amino acid sequences of fish species was observed in BLAST search and multiple alignment. Among the fish species, separate subgrouping was reasonable within the members of the *Cyprinidae* family.
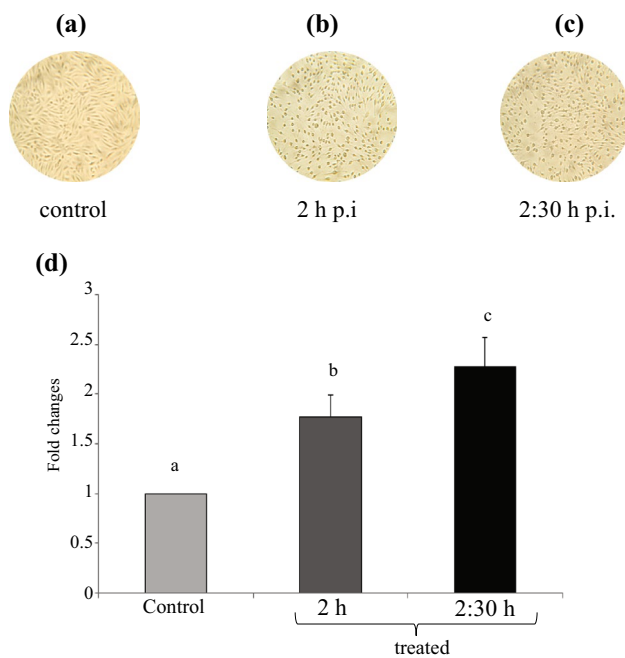
Fig. 8 Lrcasp9 is induced during cell death following *A. hydrophila* infection. *L. rohita* gill (LRG) cell line was cultured for 24 h and was either mock infected or infected with *A. hydrophila* (at 1 M.O.I) for different time intervals. The cells were observed under inverted microscope (×10) to visualize the morphological changes of the control (**a**), at 2-h infection (**b**) and at 2.30-h infection (**c**). From mock infected and *A. hydrophila* infected cells, total RNA was extracted and quantitative real-time PCR was conducted to analyze caspase-9 and β-actin gene expression (**d**). Data were normalized with β-actin and expressed as fold changes relative to the control. The results were expressed as mean ± standard errors (bars)

Basal expression profile of Lrcasp9 gene revealed constitutive expression in all tested tissues with blood expressed highest amount while kidney followed by gill the lowest. This expression pattern was partly similar to sea bass (Reis and Vale Ado 2007), human (Srinivasula et al. 1996; Renatus et al. 2001; Angelastro et al. 2001) and large yellow croaker (Mu et al. 2010) in some tissues and was different in some tissues. The difference in Lrcasp 9 basal expression profile in various tissues compared to other fishes might be due to the variation in fish species.

To investigate the modulation of Lrcasp9 gene expression in pathogenic invasion, rohu fingerlings were infected with *A. hydrophila* or *E. tarda*, common pathogens causing diseases in rohu in freshwater aquaculture (Karunasagar et al. 1989; Shome et al. 1996). In response to *A. hydrophilla* infection, tissue-specific differential expression of caspase-9 was noted in gill, liver and kidney at 24–48 h post-infection and then decreased to basal level at 72 h. In *E. tarda* infection, similar pattern of increased in caspase-9 transcript was noted in gill, liver, kidney and spleen. In sea bass, similar observation was also noted in kidney

following *Photobacterium damselae ssp. piscicida* (*Phdp*) strain PP3 infection (Reis and Vale Ado 2007). With the advancement of time, mRNA gets translated into protein resulting in decrease in mRNA transcripts and increase in protein quantity. Therefore, in the treated fish tissues possibly enhanced quantity of Lrcasp9 mRNA was noted at the earlier time points and was gradually decreased with the advancement. In mammals, enhanced expression of the initiator caspase-9 indicated the activation of intrinsic apoptotic pathway (Hakem et al. 1998; Boatright and Salvesen 2003). A similar phenomena of enhanced caspase-9 gene expression in pathogenic stimuli perhaps also indicate the activation of intrinsic apoptotic pathway in *L. rohita*.

Various fish species are infected with the members of the rhabdoviridae family, viz., viral hemorrhagic septicemia virus (VHSV), hirame rhabdovirus (HIRRV), snakehead rhabdovirus (SHRV), infectious hematopoietic necrosis virus (IHNV), pike fry rhabdovirus (PRV), spring viremia of carp virus (SVCV), starry flounder virus and ulcerative disease rhabdovirus (UDRV) (Mork et al. 2004; Whitfiel et al. 2011). Among these, VHSV, IHNV and SVCV are described as dreaded fish pathogens causing extensive morbidity and mortality in both wild and cultured fish (Bootland and Leong 1999). SVCV primarily affects cyprinid fishes (Ahne et al. 2002) but the host range of VHSV is very wide (Al-Hussinee et al. 2011). To understand the response of Caspase-9 in rhabdoviral infections, inactivated rhabdoviral vaccines of animals were injected into rohu fingerlings (Basu et al. 2016; Banerjee et al. 2017) and the data revealed significant increase in caspase-9 gene expression in gill, liver and spleen. In large yellow croaker, caspase-9 gene expression was also induced in spleen and kidney following poly I:C or bacterial vaccine stimulation, and it was highest at 48 h and then decreased at 72 h (Mu et al. 2010). Together, these data suggest a critical role of caspase-9 in bacterial and viral infection. To further investigate the expressional modulation of Lrcasp9 gene, LRG cell line was infected with *A. hydrophila* and time-dependent expression of caspase-9 gene was analyzed by qRT-PCR assay. Progression of apoptosis and cell death with the advancement of time was confirmed under microscopical observation, and the inductive expression of caspase-9 gene by qRT-PCR. In mammals, induction of caspase-9 indicated the activation of intrinsic apoptotic pathway (Hofmann et al. 1997; Boatright and Salvesen 2003). In rohu, we detected inductive expression of caspase-9 gene following in vivo and in vitro infection, which may suggest the activation of intrinsic apoptotic pathway during pathogenic invasion. Further studies are required to investigate the detail mechanism of caspase-9 in inducing apoptosis in fish.

In conclusion, caspase-9 full-length cDNA of the Indian major carp (IMC) rohu (Lrcasp9) has been cloned and its structural relationship with other fish species, mouse and

human has been studied showing the presence of evolutionary-conserved motifs from fish to human. The basal expression profile of Lrcasp9 gene has been studied in various organs/tissues showing its highest expression in blood. Following bacterial infections and anti-viral vaccinations, Lrcasp9 gene expression has been studied showing its significantly enhanced expression in most of the organs/tissues. These results together may suggest the activation of intrinsic apoptotic pathway in *L. rohita* following pathogenic stimuli as reported in higher vertebrates.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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