Journal of Aquaculture Research & Development

Mary Lini et al., J Aquac Res Development 2013, 4:6
DOI: 10.4172/2155-9546.1000200

Research Article Open Access

Effect of *Aeromonas hydrophila* Infection on Caspase-3 Expression and Activity in Rohu, *Labeo rohita*

Mary Lini R, Pani Prasad Kurcheti*, Gireesh Babu and Purushothaman CS

Division of Aquatic Environment and Animal Health Management, Central Institute of Fisheries Education, Mumbai, India

Abstract

Caspases are aspartic acid proteases which takes part an important role in the apoptosis process. There are two groups of caspases taking part in apoptosis, as initiators and executioners. Caspases 2, 8, 9 and 10 are initiators and caspases 3 and 6 are executioners. The sequence of *Labeo rohita* caspase-3 has been identified and it was found to have highest (82%) homology with *Danio rerio* (zebra fish) followed by *Tanichthys albonubes* and *Gobio gobio*, all of which are freshwater fishes and belong to family Cyprinidae. The *A. hydrophila* infected tissues showed a increase in the expression of caspase 3 from 0 to 6 h in gill, liver and kidney and thereafter it reduced almost to the initial level at 24 h. The expression pattern of apoptotic genes like caspase-3 in the present study suggests that *Aeromonas hydrophila* uses a different mechanism to evade host immune response by inducing phagocyte suicide by apoptotic caspases.

Keywords: Caspase-3; *Labeo rohita*; Apoptosis; *Aeromonas hydrophila*

Introduction

Aquaculture is a fast growing food production system with an average compounded rate of 9.6% annually. Carps account over 80% of the farmed fish production in India [1]. *Labeo rohita* is the most preferred commercial carp in India because of its qualities like effective utilization of natural food and high food conversion efficiency, acceptance of artificial feed, compatibility with other species cultured, non-predatory and able to breed by inducement. *Labeo rohita* is considered as the tastiest fish in India [2]. Among different types of infectious agents, bacterial pathogens are often responsible for severe mortalities in a wide range of fishes at different stages of growth [3,4]. *Aeromonas hydrophila*, a serious pathogen of Rohu causes hemorrhagic septicaemia, dropsy, fin and tail rot and ulcers [5]. Bacterial pathogens have developed different strategies to survive inside the host and to overcome their natural defenses, and thus cause disease. So, the induction of host cell apoptosis by bacteria is considered an important mechanism for counteracting host immune defenses [6,7]. Caspases are aspartate-specific cysteine proteases [8]. Caspase-3 is a 36 kDa zymogen [9]. Activated caspase-3 is a central effector of apoptosis that cleaves and inactivates a number of molecules contributing to the typical morphology of apoptosis [10,11]. Increased gene expression of caspase-3 has been described in cells under apoptosis [12-14]. The present study was aimed to determine the fate of caspase-3 gene expression in *Labeo rohita* upon infection with *Aeromonas hydrophila*, which is a serious pathogen of Rohu.

Materials and Methods

Experimental animals and design

Freshwater fish, *Labeo rohita* was used for the study. The fishes were procured from a local fish farm near Panvel, Mumbai. Fishes of approximately 20 g in weight were used for the experiment. The experimental setup consisted of four tanks, one control tank and three replicates for artificial infection with *A. hydrophila*. Each tank had 20 fishes. The bacterial injection was given in the morning and the sampling was done at different time intervals such as 0, 6, 12 and 24 hours post infection. The caspase-3 expression analysis was performed in three different tissues namely gills, liver and kidney (Figure 1).

Bacteria

Aeromonas hydrophila, pathogenic to fish was used for the study. The bacterial strain available in the lab was used after confirming the species with selective agar plating. 0.1 ml (1×10^9 CFU/ml) of bacterial culture per 10 g body weight was administered intraperitoneally for inducing artificial infection.

Tissue collection and preservation

The sampling was done at different time intervals such as 0, 6, 12 and 24 h post infection. Different tissues *viz* gills, liver and kidney were collected using sterile scissors and forceps. Each specimen was preserved in RNA later, for further use.

Identification of Rohu caspase 3

Total RNA was extracted using Trizol reagent (Invitrogen, USA) following the manufacturer's instructions and the RNA pellet was dissolved in 30 µl of nuclease free water. The quality of RNA was confirmed by gel electrophoresis, and the quantity was obtained by using Bio Photometer (Eppendorf). The extracted RNA was treated with DNAse I (RNase free) to remove the genomic DNA contamination. The template RNA (2 µg) was converted into cDNA by Revert Aid H minus MMuLV reverse transcriptase (Invitrogen, USA) with oligo(dt) primer.

PCR reaction was performed with the degenerate primers for caspase-3 and specific primers for β-actin. The primers used were Lr Actin-F GGGTATGGAGTCTTGCGGTATC, Lr Actin-R CACATCTGCTGGAAGGTGGAC for amplifying β-actin gene and

***Corresponding author:** Pani Prasad Kurcheti, Division of Aquatic Environment and Animal Health Management, Central Institute of Fisheries Education, Mumbai, India, Tel: +91-22-26361446; E-mail: pprasad35@rediffmail.com; kpaniprasad@cife.edu.in

Received July 27, 2013; **Accepted** October 25, 2013; **Published** October 31, 2013

Citation: Mary Lini R, Kurcheti PP, Babu G, Purushothaman CS (2013) Effect of *Aeromonas hydrophila* Infection on Caspase-3 Expression and Activity in Rohu, *Labeo rohita*. J Aquac Res Development 4: 200 doi:10.4172/2155-9546.1000200

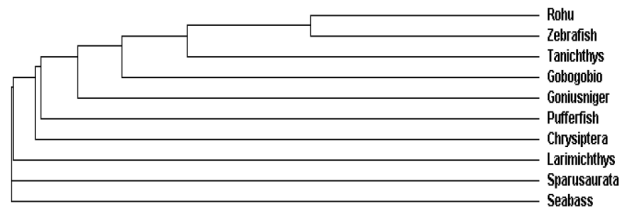


Figure 1: Phylogenetic tree constructed based on the Caspase 3 sequences from different fish species.

DegnCasp3-F ATCATHAAYAAYAARAAYTTYCA and DegnCasp3-R CAGRAARTCNGCYTCNACNGGVAT for caspase 3 gene. For caspase-3, the reaction conditions used were as follows. Initial denaturation at 94°C for 3 min, then 15 cycles of 94°C 30 sec, 45°C 1 min, 72°C 1 min with 1 degree increment in annealing temperature per cycle, followed by 25 cycles of 94°C 30 sec, 60°C 45 sec, 72°C 1 min and the final extension was carried out at 72°C for 8 min. The cycling reaction was performed in C1000 Thermocycler (BioRad). Agarose gel electrophoresis using 1.8% agarose gel was used for analysing the amplified products of PCR. The gel was then analyzed using the gel documentation system (DNr Bioimaging Systems, Israel).

The bands obtained in the gel were cut and DNA eluted using quick Gel extraction kit (Qiagen) following manufacturer's instructions. The eluted DNA was T/A cloned into PTZ57R vector using T4 DNA ligase and transformed into DH5α *E. coli* strain. The positive clones were identified.

The plasmid DNA was isolated from the positive colonies using Gene JET plasmid miniprep kit (Fermentas) following manufacturer's instructions. Restriction enzyme digestion was done for the recombinant plasmid to confirm the presence of insert. The plasmids that released the desired inserts were confirmed as positive clones.

Caspase 3 gene sequencing and analysis

The cloned product was sequenced using M13 universal primers by Bioserve, Hyderabad, India. The sequence obtained was subjected to BLASTn homology search to confirm the sequence. The top 10 sequences were taken for multiple sequence alignment using ClustalW software and phylogenetic tree was constructed. The Generunner software was used to translate the sequence into protein sequence to analyse the amino acid sequence for the presence of functional domains that are specific to caspase-3.

Semi quantitative RT-PCR analysis of Rohu caspase-3

The semi quantitive analysis of Rohu caspase-3 expression at different time intervals post infection (0, 6, 12 and 24 h) was studied by RT-PCR analysis. The PCR conditions were used as mentioned above. β-actin was used as internal control to normalize the expression levels.

Results and Discussion

Molecular characterization of caspase 3 of *Labeo rohita*

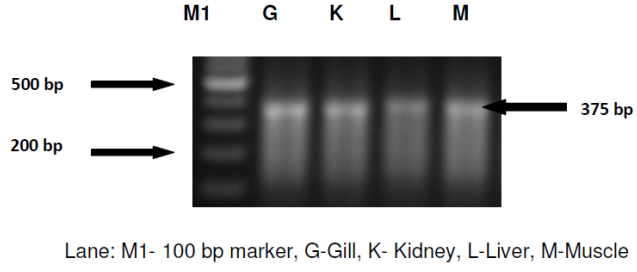
Natural selection has a key role in determining how each pathogen inhibits apoptosis. As the host evolves to eliminate a bacterial infection, the pathogen must itself evolve to counteract this change to maintain the replicative niche inside the host. Bacteria, viruses [15] and parasites [16] can either induce or prevent apoptosis to augment infection. Many bacterial pathogens that cause apoptosis target immune cells such as macrophages [17] and neutrophils [18] because these cells would otherwise kill the pathogens [19,20].

On the other hand *S. flexneri* might block apoptosis by targeting the activated form of caspase-9 or the inactive form of caspase-3 to prevent caspase-3 activation. Apoptosis inhibition during early infection despite caspase-3 activation is achieved through the activation of NF-kB, which is also dependent on the Dot/Icm system [21]. Besides activation of caspase-3 zymogen, increased gene expression of caspase-3 was described in cells undergoing apoptosis. In the *in vitro* tests, the caspase-3 mRNA expression of haemocytes significantly increased at 30 min, reached the highest value at 60 min, and then began to return to the control level after 120 min. These facts suggest that the stress-inducing neutrophil elastase upregulated the transcription of caspase-3 mRNA and increased caspase-3 activity, which promoted the occurrence of haemocyte apoptosis [12]. Therefore the present study was aimed to determine the fate of caspase-3 gene expression in *Labeo rohita* upon infection with *Aeromonas hydrophila*, which is a serious pathogen of Rohu.

PCR analysis of the cDNA prepared from liver, kidney and gills using caspase-3 degenerate primers resulted in a 375 bp product (Figure 2). The caspase-3 gene has been sequenced and characterized in zebrafish (*Danio rerio*) [22-24], sea bass (*Dicentrarchus labrax L.*) [25] and gudgeon (*Gobio gobio*) [26], though sequences are available in the databases for other teleostei species, such as trout (*Oncorhynchus mykiss*), salmon (*Salmo salar*), seabream (*Sparus auratus*), Japanese medaka (*Oryzias latipes*), channel catfish (*Ictalurus punctatus*), mandarin fish (*Siniperca chuatsi*) and tiger puffer (*Takifugu rubripes*). The BLAST search at NCBI resulted in the caspase-3 sequences reported from several fish species. The caspase-3 sequence generated in the present study showed highest homology of 82% with zebrafish (Genbank) and followed by 67% homology with *Tanichthys albonubes.*

Phylogenetic analysis of caspase-3 gene from various fishes showed the cloned fish caspase 3 members are 50-70% identical with those of non-fish vertebrate species [27]. The identities of the caspase-3 of zebrafish with chicken, hamster, human, rat, mouse and *Xenopus* caspase-3 were 64, 62, 62, 62, 61 and 58% respectively. Partial caspase-3 sequence of gudgeon shares an identity of 82% with *D. rerio* (DQ812120), 72% with *S. salar* (BT044884) and 69% with *C. parasema* (DQ073799).

The amino acid sequence obtained was analyzed for the presence of functional domains that are reported to be present in other vertebrate species. It was revealed that Rohu caspase 3 partial amino acid sequence also had the pentapeptide active site motif QACRG along with highly conserved 'RGD' motif (Figure 3). These results suggest that the functional domains that are known to be conserved in other vertebrate species are also conserved in *Labeo rohita* too. Several authors have



Lane: M1- 100 bp marker, G-Gill, K- Kidney, L-Liver, M-Muscle

Figure 2: RT- PCR amplification of caspase-3 cDNA of *Labeo rohita.*

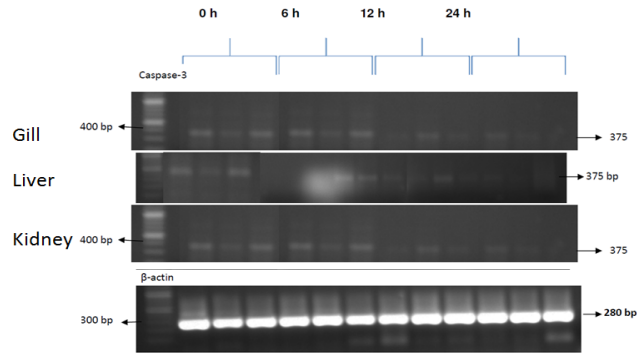


Figure 3: Caspase-3 and β-actin expression levels in gill, liver and kidney tissue after infection with *A. hydrophila*.

IINKKNFDRRTSMNHANGTEERCSSNFNVFRAFFIVGLQLHQ
TVVRRMQVSNSCRHDDHPAVLINVMLSHRDERVLNGNDFIS
WIKS**RGD**SSSEARPASLFGRASLIY**QACRG**TEMDPKVNKTSL
RHCHQLSSLKDQCRQISS

Figure 4: Identification of functional domains in the translated amino acid sequence of Rohu Caspase 3 cDNA.

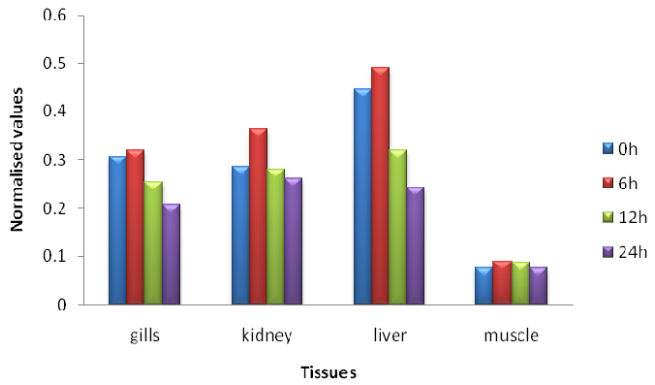


Figure 5: Caspase 3 expression levels in *L. rohita* at different time intervals after artificial infection with *A. hydrophila*.

reported the presence of these functional domains in other fish species including mammals. The pentapeptide active site motif (QACRG) and the putative aspartic acid (Asp 182) are known to split the large and small subunits of pro-caspase 3 in to two making it active. Therefore, this is obvious that the particular motif will be conserved across taxa [25,28,29]. Also an integrin recognition motif (RGD) [29,30] near the active site was found to be conserved in all species analyzed with the exception of sea bass, where the aspartate residue is replaced by an asparagine (N153) [25].

Rohu caspase 3 gene expression upon infection

Once the partial sequence has been characterized the expression levels of caspase-3 have been determined in different organs of Rohu subjected to artificial infection with *A. hydrophila*. The caspase-3 expression levels were analyzed at 0, 6, 12 and 24 hours post infection in liver, kidney, gill and muscle using semi quantitative RT-PCR technique. The caspase-3 expression levels were normalized with β-actin as internal control to avoid any errors in the initial RNA concentration. From the results obtained it was found that in the gill, kidney and liver tissue the expression levels showed a decrease from 6 h to 12 h and continued to be lower in 24 h (Figures 4 and 5).

The key virulence mechanism of several bacteria is through induction of apoptotic pathway through caspase-3. In this case, it is obvious that upon bacterial infection the caspase-3 mRNA levels should shoot up in order to meet the increasing demand for the execution of apoptosis. Unexpectedly, the down regulation of the apoptotic caspases like caspase-3 as observed in the present study suggests a different mechanism that *Aeromonas hydrophila* employs to evade host immune response by inducing phagocyte survival through the infection of apoptotic caspases. It has been reported that apoptosis of the infected cells can limit the spread of intracellular micro organisms by provoking inflammatory responses as a compensatory mechanism for the removal of these cells through the recruitment of phagocytes [31]. However a variety of mammalian pathogens are capable of surviving inside host cells by interfering with host cell apoptotic processes [32-34]. A similar kind of observation has been reported by Sepulcre et al. [35] who suggested that *Vibrio anguillarium* evades the immune response of the bony fish sea bass (*Dicentrachus labrax*) through the down regulation of apoptotic caspases. However, further studies need to be conducted to confirm whether the same mechanism is being operated by *Aeromonas hydrophila* in Rohu.

In conclusion, the partial sequence (caspase 375 bp) of the *Labeo rohita* caspase-3 gene has been characterized, which showed highest homology (82%) with zebrafish followed by *Tanichthys albonubes* (67%). The expression analysis showed that there is a decrease in caspase-3 levels in liver and kidney six hours post infection, which throws light on the virulence mechanism of *Aeromonas hydrophila* in Rohu. However, further studies are required before establishing the virulence mechanism of *Aeromonas hydrophila*.

Acknowledgement

The authors acknowledge with thanks Dr. Lakra WS, Director/Vice Chancellor, Central Institute of Fisheries Education, Mumbai, India for the help and facilities provided in completion of the research work.

References

1. Gupta S, Gupta P (2006) General and Applied Ichthyology. S. Chand and Company Ltd 708-715.
2. Reddy SM, Rao DS (1999) Taxonomy, In: A textbook of aquaculture. Discovery publishing house 1-14.
3. Grisez L, Ollevier FP (1995) Vibrio (Listonella) anguillarum infections in marine fish larviculture, In: Lavens P (ed.) Larvi '95: Fish & Shellfish Symposium. EAS Special Publication, Gent, Belgium, 24: 497.
4. Swain P, Nayak SK, Sahu A, Mohapatra BC, Meher PK (2002) Bath immunization of spawns, frys and fingerlings of Indian major carps using a particulate antigen. Fish Shellfish Immunol 13: 133-140.
5. Rahman MH, Suzuki S, Kawai K (2001) The effect of temperature on Aeromonas hydrophila infection in goldfish, Carassius auratus. J Appl Ichthyol 17: 282-285.
6. Moss JE, Aliprantis AO, Zychlinsky A (1999) The regulation of apoptosis by microbial pathogens. Int Rev Cytol 187: 203-259.
7. Weinrauch Y, Zychlinsky A (1999) The induction of apoptosis by bacterial pathogens. Annu Rev Microbiol 53: 155-187.
8. Alnemri ES, Livingston DJ, Nicholson DW, Salvesen G, Thornberry NA, et al. (1996) Human ICE/CED-3 protease nomenclature. Cell 87: 171.
9. Deveraux QL, Roy N, Stennicke HR, Van Arsdale T, Zhou Q, et al. (1998) IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. EMBO J 17: 2215-2223.

10. Fischer U, Jänicke RU, Schulze-Osthoff K (2003) Many cuts to ruin: a comprehensive update of caspase substrates. Cell Death Differ 10: 76-100.

11. Wolf BB, Green DR (1999) Suicidal tendencies: apoptotic cell death by caspase family proteinases. J Biol Chem 274: 20049-20052.

12. Chiang LW, Grenier JM, Ettwiller L, Jenkins LP, Ficenec D, et al. (2001) An orchestrated gene expression component of neuronal programmed cell death revealed by cDNA array analysis. Proc Natl Acad Sci U S A 98: 2814-2819.

13. Miller TM, Moulder KL, Knudson CM, Creedon DJ, Deshmukh M, et al. (1997) Bax deletion further orders the cell death pathway in cerebellar granule cells and suggests a caspase-independent pathway to cell death. J Cell Biol 139: 205-217.

14. Yakovlev AG, Ota K, Wang G, Movsesyan V, Bao WL, et al. (2001) Differential expression of apoptotic protease-activating factor-1 and caspase-3 genes and susceptibility to apoptosis during brain development and after traumatic brain injury. J Neurosci 21: 7439-7446.

15. Hay S, Kannourakis G (2002) A time to kill: viral manipulation of the cell death program. J Gen Virol 83: 1547-1564.

16. Bruchhaus I, Roeder T, Rennenberg A, Heussler VT (2007) Protozoan parasites: programmed cell death as a mechanism of parasitism. Trends Parasitol 23: 376-383.

17. Zhang Y, Ting AT, Marcu KB, Bliska JB (2005) Inhibition of MAPK and NF-kappa B pathways is necessary for rapid apoptosis in macrophages infected with Yersinia. J Immunol 174: 7939-7949.

18. Blomgran R, Zheng L, Stendahl O (2004) Uropathogenic Escherichia coli triggers oxygen-dependent apoptosis in human neutrophils through the cooperative effect of type 1 fimbriae and lipopolysaccharide. Infect Immun 72: 4570-4578.

19. Kobayashi SD, Braughton KR, Whitney AR, Voyich JM, Schwan TG, et al. (2003) Bacterial pathogens modulate an apoptosis differentiation program in human neutrophils. Proc Natl Acad Sci U S A 100: 10948-10953.

20. Grassmé H, Jendrossek V, Gulbins E (2001) Molecular mechanisms of bacteria induced apoptosis. Apoptosis 6: 441-445.

21. Abu-Zant A, Jones S, Asare R, Suttles J, Price C, et al. (2007) Anti-apoptotic signalling by the Dot/Icm secretion system of L. pneumophila. Cell Microbiol 9: 246-264.

22. Yabu T, Kishi S, Okazaki T, Yamashita M (2001) Characterization of zebrafish caspase-3 and induction of apoptosis through ceramide generation in fish fathead minnow tailbud cells and zebrafish embryo. Biochem J 360: 39-47.

23. Chakraborty C, Nandi SS, Sinha S, Gera VK (2006) Zebrafish caspase-3: molecular cloning, characterization, crystallization and phylogenetic analysis. Protein Pept Lett 13: 633-640.

24. Eimon PM, Ashkenazi A (2010) The zebrafish as a model organism for the study of apoptosis. Apoptosis 15: 331-349.

25. Reis MI, Nascimento DS, do Vale A, Silva MT, dos Santos NM (2007) Molecular cloning and characterisation of sea bass (Dicentrarchus labrax L.) caspase-3 gene. Mol Immunol 44: 774-783.

26. Nadzialek S, Pigneur LM, Wéron B, Kestemont P (2010) Bcl-2 and Caspase 3 mRNA levels in the testes of gudgeon, Gobio gobio, exposed to ethinylestradiol (EE2). Aquat Toxicol 98: 304-310.

27. Chowdhury I, Tharakan B, Bhat GK (2008) Caspases-an update. Comp Biochem Physiol B Biochem Mol Biol 151: 10-27.

28. Pasqualini R, Koivunen E, Ruoslahti E (1995) A peptide isolated from phage display libraries is a structural and functional mimic of an RGD-binding site on integrins. J Cell Biol 130: 1189-1196.

29. Ruoslahti E (1996) RGD and other recognition sequences for integrins. Annu Rev Cell Dev Biol 12: 697-715.

30. Pierschbacher MD, Ruoslahti E (1984) Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. Nature 309: 30-33.

31. Williams GT (1994) Programmed cell death: a fundamental protective response to pathogens. Trends Microbiol 2: 463-464.

32. Rosenberger CM, Finlay BB (2003) Phagocyte sabotage: disruption of macrophage signalling by bacterial pathogens. Nat Rev Mol Cell Biol 4: 385-396.

33. Zhang JZ, Sinha M, Luxon BA, Yu XJ (2004) Survival strategy of obligately intracellular Ehrlichia chaffeensis: novel modulation of immune response and host cell cycles. Infect Immun 72: 498-507.

34. Marriott HM, Hellewell PG, Cross SS, Ince PG, Whyte MK, et al. (2006) Decreased alveolar macrophage apoptosis is associated with increased pulmonary inflammation in a murine model of pneumococcal pneumonia. J Immunol 177: 6480-6488.

35. Sepulcre MP, López-Castejón G, Meseguer J, Mulero V (2007) The activation of gilthead seabream professional phagocytes by different PAMPs underlines the behavioural diversity of the main innate immune cells of bony fish. Mol Immunol 44: 2009-2016.