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EXPRESSION PATTERN OF BETA DEFENSIN-3 IN THE SMALL INTESTINE OF ZEBRA FISH *DANIO RERIO*

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Keywords:

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ABSTRACT: The vertebrate immune system is composed of numerous distinct and interdependent components, and it consists of both systemic and mucosal immune compartments, but the mucosal immune system which protects the body from the first encounter of pathogens. β-defensins are a group of cysteine-rich cationic antimicrobial peptides that play an antimicrobial roles in the immune systems of vertebrates. Vertebrate defensins can be classified into α-, β, and θ-defensins based on their cysteine disulphide bonding. In the present study, we report the expression level of the β-defensin- 3 in the small intestine of the zebrafish after a bacterial challenge with Staphylococcus aureus with 100% and 50% concentrations. The results revealed that the β-defensin-3 was highly upregulated in both 100% and 50% concentration of the bacterial pathogen, and it plays an important role in the innate and mucosal immunity in zebrafish.

INTRODUCTION: Defensins are a group of small, cationic, amphipathic, cysteine-rich anti-microbial peptides (AMPs) that have important effects in the host innate immune systems of various organisms [1]. Defensin in animals contains six cysteines that form three intracellular disulphide bonds and a β-sheet structure. Vertebrate defensins can be classified into α-, β-defensins on their cysteine disulphide bonding. According to the Food and Agriculture Organization of the United Nations (FAO), aquaculture production has increased from 9% of the fisheries resources in 1980 to 43% at present, and it is thought that production will need to be double in the next 25 years [2].

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Over the past decade, the challenges in large-scale production of aquatic animals are exposed for stressful conditions, problems related to diseases, and deterioration of environmental conditions [3]. The major challenges for the aquaculture industry have been the prevalence of diseases, which results in severe annual economic losses [4]. Control of such pathogens (most of which are bacterial) in the fish farm has been routinely achieved by the administration of antimicrobial agents.

However, the excessive use of these antimicrobials has led to the emergence of antibiotic-resistant bacteria, due to those drug-resistant strains carrying a transferable R-plasmid, making the treatments less successful. Several investigations have been reported that AMPs exhibit antimicrobial activity and an immune-modulatory effect, which enhances host defensive mechanisms against infections [5]. These biological functions suggest a potential value of AMPs in aquaculture applications, and accelerated efforts to clone teleost fish genes

encoding AMPs have been initiated. Defensins are hypothesized to have originated in prokaryotes and then diverged into plants followed by arthropods [6, 7]. They can rapidly kill pathogens, including bacteria [9], mycobacteria, fungi [10,] and viruses [11]. β-defensins have been revealed to have more biological activities such as broad-spectrum of antimicrobials [8]. β-defensins have been found in various vertebrates.

Including bovine, human, mouse, birds, reptiles, and fishes [12], while α- defensins are known only in mammals and suggesting that the defensin family has expanded throughout evolution and that all the subfamilies may have arisen from an ancestral β-defensin gene by duplication and diversification.

In a healthy fish, some of these β-defensins are expressed in a wide range of mucosal and systemic tissues, including the gills, gonad, gut, peritoneal leucocytes, kidney, liver, muscle, skin, and spleen [13]. The immune system of vertebrates is composed of two subdivisions, the innate and the adaptive immunity. The innate immune system provides the first line of defense against invading pathogens. In fish, the adaptive immune system is less developed; hence innate system is vitally important, and the release of various AMPs is a major mechanism in the innate immune system [14].

MATERIALS AND METHODS:

Fish Rearing: *Danio rerio* was purchased from an aquarium in Coimbatore; the fishes were brought to the laboratory and acclimatized for a period of one week. The fishes were fed regularly with artificial feed.

Bacterial Challenge: For the bacterial challenge, the culture of Staphylococcus aureus was purchased from the Microbiological laboratory and as incubated at C overnight in Luria- Bertani medium containing 3% NaCl in an orbital shaker. Three adult healthy *Danio rerio* species were injected intraperitoneally with 20 µl of *Staphylococcus aureus*, 100 mg/g, and 50 mg/g per fish, and the control was injected with Phosphate buffer solution. After the challenge, fishes were placed in a separate rectangular tank of freshwater. At 18th post-injection, all the fishes were euthanized, and the small intestine was sampled for total RNA extraction, while the three unchallenged fish served as control.

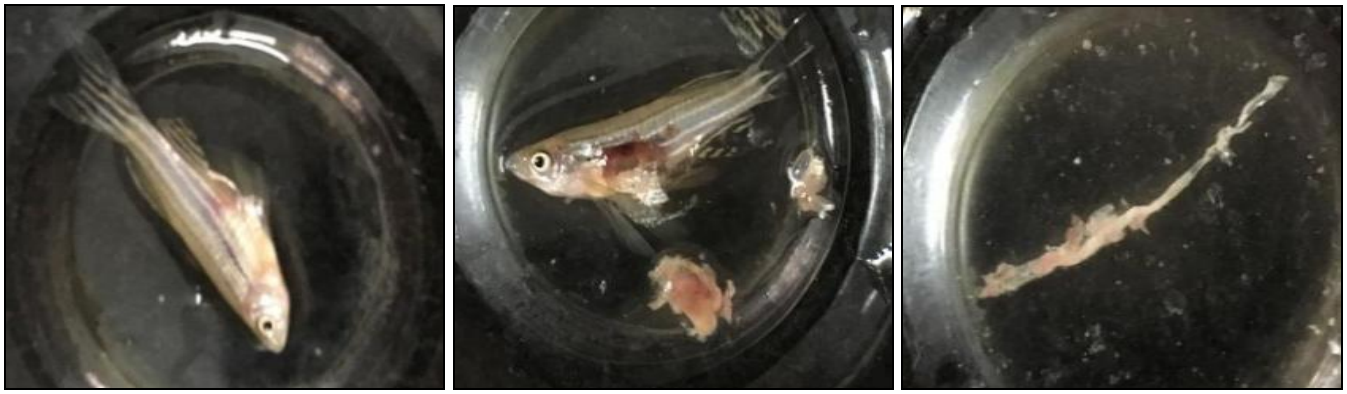

FIG. 1: THE DISSECTION OF SMALL INTESTINE IN THE ZEBRA FISH *DANIO RERIO*

CDNA Extraction: About 10-20 mg of the fish intestine was finely chopped with a sterile spatula and then grinded using mortar and pestle with pre-chilled liquid nitrogen. The minced tissue was then transferred to a microfuge containing 600 µl of ice-cold lysis buffer.

The suspension was quickly homogenized with 30-50 strokes of a microfuge pestle (Optional add 3 µlproteinase K solution to the lysate to increase the yield of genomic DNA). The digest was then incubated for at least 3 h at 55 ºC in a shaking incubator. It was then allowed to cool to room temperature and added 3 µl of 4 mg/ml, DNase free RNase and incubated for 15 min at 37 ºC. The samples were cooled to room temperature and 200 µl of 5 M sodium acetate and mixed the contents by vortexing vigorously for 20 seconds.

The precipitated protein and SDS were pelleted by centrifugation at 4000 rpm for 3 min at 4 ºC. The supernatant was transferred to a fresh microfuge tube containing 600 µL of isopropenol, and the solution was then mixed well to recover the

precipitate of DNA and centrifuging it for 1 min at 14000 rpm in room temperature. The supernatant was removed by aspiration, and 600 µl of 70% ethanol was added to the DNA pellet. The tube was inverted several times and centrifuged at 4000 rpm for 1 min at room temperature. The supernatant was removed carefully by aspiration and allowed the pellet to dry for 15 mim, and the pellet was resuspended in 50 µL of TE-buffer [15].

RT-PCR: Amplification of the β-defensin-3 c-DNA fragments in the zebrafish was done by RT-PCR and primers were designed based on the cDNA sequence of zebrafish β-defensin-3. PCR was performed with the following settings with denaturation at 94 ºC for 5 min followed by 30 cycles of 94 ºC for 30 seconds, annealing for 45 seconds, at 72 ºC for 1 min, with a final extension step of 72 º C for 10 min. PCR products were detected on 1% agarose gel, and the anticipated fragments were purified from the gel. The real-time PCR analysis of β- defensin 3 gene expressions was done with SYBR green real master mix with a 125-real-time PCR instrument. The amplification scheme was incubated for 1 min at 94 ºC, followed by 40 cycles for 20 seconds at 94 ºC, 20 seconds for annealing, and 50 seconds at 70 ºC. For each mRNA gene expression was corrected by 40 s ribosomal protein in each sample, and the relative expression of the zebrafish β-defensin-3 mRNA was determined in triplicate samples.

TABLE 1: THE PRIMERS USED FOR THE RT-PCR PROGRAMME

β-Actin	ACCTGACAGA CTACCTGATG	TGAAGGTGGTCTC ATGGATAC
β-Defensin-3	GAAAACTGGA GCTCCTGATC	ATGCTCTGGGGCT TCATGTT

RESULTS: In the present study, the expression level of the gene β-defensin-3 was analyzed from the small intestine of zebrafish by Trizol method. For the experimental study, we used β-actin as the control gene and β-defensin as the experimental gene. The β-actin is one of the six different isomers and a cycloskeletal protein involved in cellular functions.

The expression level of β- defensin-3 was analyzed after a bacterial challenge of *Staphylococcus aureus*, which was injected intraperitoneally with 100% and 50% concentration, and the other group was kept it as the control. In this study, the induction level occurred at 100% concentration was 0.45 fold, and at 50%, it was 0.18 fold after the bacterial infections **Table 2** & **Fig. 2**. These results indicated that the β-defensin -3 was highly expressed in the 100% concentration when compared to the 50% concentration. This indicated that the gene β- defensin shows more susceptibility towards the high bacterial concentration.

The wide expression of β-defensin 3 in the small intestine of zebrafish plays an important role in the innate immune response against bacterial infections. The expression level of β-defensin- 3 in the zebrafish after the intraperitoneal injection with *Staphylococcus aureus* was up-regulated.

The expression of β-defensin-3 in the small intestine of the zebrafish was induced markedly in the present study, and the highest induction level occurred at the 18th post hour after infection. The up-regulation in the systemic immune organs of the zebrafish suggests that β-defensin-3 helps the fish in defense mechanisms.

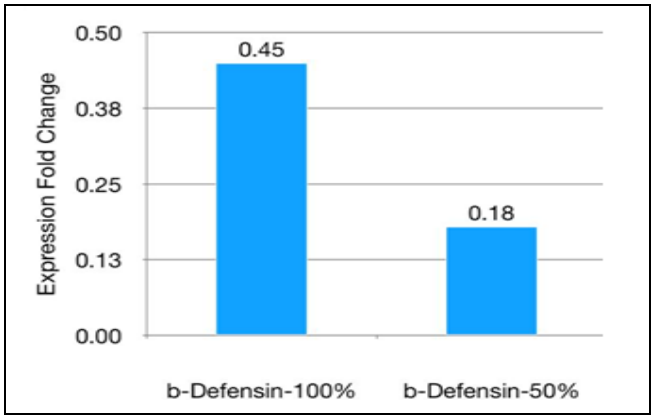

FIG. 2: THE EXPRESSION FOLD CHANGE OF THE ZEBRA FISH WITH 100% AND 50% BACTERIAL (*STAPHYLOCOCCUS AUREUS*) CONCENTRATION

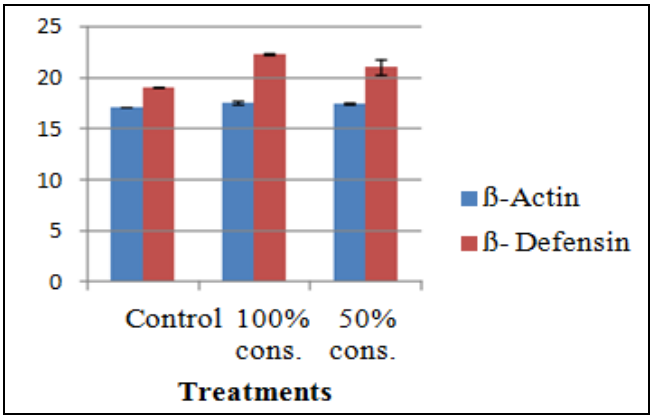

FIG. 3: THE B-DEFENSIN 3 GENE IN COMPARISON WITH HOUSE KEEPING GENE ACTIN

TABLE 2: EXPRESSION OF B-DEFENSIN-3 IN THE SMALL INTESTINE OF *ZEBRA FISH*

	Experimental			Control			Average Experimental Ct Value	Average Experimental Ct Value	Average Control Ct Value	Average Control Ct Value	ΔCt Value (Experimental)	ΔCt Value (Control)	Delta Delta Ct Value	Expression Fold Change
	1 Raw Ct Value	2 Raw Ct Value	3 Raw Ct Value	1 Raw Ct Value	2 Raw Ct Value	3 Raw Ct Value	TE	HE	TC	HC	ΔCTE	ΔCTC	ΔΔCt	2^-ΔΔCt
Untreated	18.56	19.13	19.42	17.06	17.38	15.61	-	19.04	-	16.68				1.00
β-Defensin-100%	21.23	19.62	22.18	17.67	17.67	17.17	21.01	-	17.50	-	1.97	0.82	1.15	0.44993 1655
β-Defensin-50%	22.19	22.183 33333	22.426 66667	17.266 66667	17.556 66667	17.483 33333	22.26666667	-	17.43555 556	-	3.23	0.752222 222	2.477777 778	0.17952 0714

(TC-Target gene in control, HC- housekeeping gene in control, CT-Delta and fold change, TE&HE- Target gene and housekeeping gene in experimental samples

TABLE 3: THE EXPRESSION LEVEL OF THE GENE B-ACTIN AND B- DEFENSIN USING DUNCAN MULTIPLE RANGE TEST (P<0.05)

Treatments	β-Actin		β- Defensin	
Control	17.06	± 0.006[b]	19.03	± 0.252[b]
100% Bacterial concentration	17.50	±0.166 [a]	22.25	±0.071[a]
50% Bacterial concentration	17.43	±0.86[a]	21.01	±0.747[a]
Significance value	0.059	±0.05	0.007	±0.007
(ANOVA) F value	4.703	± 4.70	12.609 ± 12.60	

DISCUSSION: The vertebrate immune system is necessary for organisms to defend themselves against invading pathogens. Every component of the immune system has its own inherent protective value, and the final combination of these components is likely to be related to a satisfactory immune response. Mucosal immunity in fish was rarely studied, although there is currently great interest in this knowledge; since the majority of the infectious agents affect body surfaces, the mucosal immune response plays a crucial role during the course of the infections [16].

Different studies have focussed on examining their cellular and molecular composition of different aquatic species in skin, gill, and gut constitute a large area for the possible invasion of pathogens, which is also influenced by the fact that there is an intimate contact between these animals and the aquatic environment.

The mucus is secreted by the globlet cells, which line the intestinal epithelium, which is composed of mucins and inorganic salts suspended in water. Mucins are a type of glycoprotein and rapidly form a gel when they leave the globlet cells and contact water. The main role of the globlet cells is to secrete mucus in order to protect the mucous membrane where they are found to repair and replace the existing mucus layer. Mucins are stored in granules inside the globlet cells before being released to the lumen of an organ [17]. Furthermore, the expression levels of β-defensin 3 mRNA in the gills, skin, foregut, and hindgut of the common carp were upregulated significantly and the highest induction levels occurred at 12 and 24 h after infections is positively correlated with present work. Hence, we speculate that some innate immune signaling pathways and induced cytokines are involved in the regulation of AMP expression in fish after bacterial challenges. In conclusion, current studies have exposed that globlet cells can obtain soluble antigens from the intestinal lumen and deliver them to subepithelial dendrite cells. Therefore, globlet cells play a part in antigen uptake and to underlying immune cells, which was previously thought to be an exclusive function of intestinal mucosal cells. The zebrafish intestine is an attractive translational model to study human diseases, and that it can also serve as the base for the fundamental research on epigenetic regulation. This study lays a solid foundation for further investigations into the possible development and application of the β-defensin gene as immune agents and for obtaining a better understanding of the immune defense mechanisms.

CONCLUSION: Fishes are the main group of vertebrates and a major component of the aquatic fauna. Although many genes have started to be studied and the cellular source(s) has not yet been determined. Utilization of these findings will improve strategies for the selection of disease-resistant broodstock and evaluation of prevention and treatment options. In the future, mucus from selected fish species may be a source of novel antimicrobial agents for human health-related

applications, besides its importance in aquaculture health and welfare. The results indicated that β-defensin-3 plays an important role in the innate immune response, especially in the hindgut and gill mucosal immune responses of the zebrafish to bacterial pathogens. The present study is helpful in protecting the fishes against many bacterial diseases in aqua farming and practices.

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CONFLICTS OF INTEREST: On behalf of all the authors, I declare that there are no known competing financial interests or personal relationships that could appear to influence the work reported in this paper.

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