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Identification and expression analysis of c-type and g-type lysozymes genes after *Aeromonas hydrophila* infection in African catfish

Identifikasi dan analisis ekspresi gen lisozim tipe-C dan tipe-G pada ikan lele dumbo setelah infeksi *Aeromonas hydrophila*

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ABSTRACT

Lysozymes play an important role in the first line of defense in fish and potentially used as an immunity status biomarker and immune responses evaluation in fish, which often found in two types, i.e. chicken-type and goose-type (c- and g-type, respectively). To recent, the information related to the sequences and the expression analysis of the c- and g-type lysozyme genes in African catfish is still limited. In the present study, we report a partial cloning and mRNA expression analysis of c-type and g-type lysozymes in African catfish *Clarias gariepinus*. We have successfully cloned and partially identify the c-type, and g-type lysozyme genes of *C. gariepinus*, which consist of 594 and 560 of coding sequences, respectively. Catalytic and other conserved residues were identified by multiple sequences alignment and they showed high similarity with other teleost fish species. mRNA levels of the genes were analyzed by using qPCR method and their expressions in the spleen, liver, and head kidney were rapidly modulated after *Aeromonas hydrophila* injection, with different patterns were observed in each organ. These results confirmed that c- and g-type lysozymes played an important role in non-specific immunity against *A. hydrophila* infection. This study provided valuable information that can be used to understand the African catfish immune systems for better disease and stress management in *C. gariepinus* culture.

Keywords: lysozymes, gene identification, gene expression, bacterial infection, African catfish

ABSTRAK

Lisozim berperan dalam sistem pertahanan dini pada ikan dan sangat potensial digunakan sebagai marka status imunitas dalam evaluasi respons imun. Lisozim umum ditemukan dalam dua tipe pada ikan: tipe-ayam (tipe-c) dan tipe-angsa (tipe-g). Informasi terkait sekuens gen dan ekspresi gen kedua tipe lisozim pada ikan lele dumbo sangat terbatas. Pada penelitian ini, kami melaporkan kloning gen secara parsial, dan analisis ekspresi gen dari kedua tipe lisozim pada ikan lele dumbo *C. gariepinus*. Sekuens parsial gen lisozim tipe-c dan tipe-g yang berhasil diidentifikasi adalah sepanjang 594 dan 560 pasang basa. Situs katalitik dan residu khas memiliki tingkat kesamaan yang tinggi dengan spesies ikan yang lain. Analisis mRNA dilakukan dengan metode *quantitative* PCR (qPCR). Ekspresi kedua gen di jaringan ginjal depan, limpa, dan hati dengan cepat terinduksi pasca infeksi bakteri *A. hydrophila* dengan pola yang berbeda. Hasil ini menunjukkan bahwa lisozim tipe-c dan tipe-g memiliki peran yang sangat penting dalam sistem imun ikan lele dumbo terhadap infeksi *A. hydrophila*. Penelitian ini menghasilkan informasi penting yang dapat digunakan untuk mempelajari sistem imun ikan lele dumbo dan sebagai acuan dalam penanganan dan manajemen penyakit pada budidaya ikan lele dumbo.

Kata kunci: lisozim, identifikasi gen, ekspresi gen, infeksi bakteri, ikan lele dumbo

INTRODUCTION

African catfish *Clarias gariepinus* is an important aquaculture species in the world that mainly produced in African and Asian country, including Indonesia (Ekasari *et al*., 2016; Shoko *et al*., 2016; Dauda *et al*., 2018). *C. gariepinus* production has increased significantly through various system development and intensive fish culture practices (Ekasari *et al*., 2016; Fauji *et al*., 2018), consequently exposes the cultured fish to common stressors including the deterioration of water quality and intensive handling. These stressors could bring about adverse effects on the fish health status and increase its susceptibility to disease infection (Cardinaud *et al*., 2014; Bardon-albaret & Saillant, 2016; Yarahmadi *et al*., 2016). One of the most commonly occurred infection diseases in African catfish is motile aeromonad septicemia (MAS) caused by *Aeromonas hydrophila* infection. This Gram-negative bacterium has caused high mortality in some freshwater aquaculture species and induced serious epidemics of ulcerative disease leading to a heavy economic loss in *C. gariepinus* culture (Angka *et al*., 1995; Azis *et al*., 2015; Zhou *et al*., 2018).

As one of the fish bacterial defense components, lysozyme has crucial roles. This hydrolase enzyme catalyzes the hydrolysis of β-(1,4)-glycosidic bond between the N-acetyl glucosamine and N-acetyl muramic acid in the peptidoglycan layer of bacterial cell walls and possess bacteriolytic activity against Gram-positive and Gram-negative bacteria (Saurabh & Sahoo, 2008; Wang *et al*., 2016). Generally, among the vertebrate and teleost fish, lysozyme is mostly found in two forms, i.e. chicken-type (*c-Lyz*) and goose-type (*g-Lyz*) (Callewaert & Michiels, 2010; Callewaert *et al*., 2012; Zhang *et al*., 2018). The c- and g-type of lysozymes have been demonstrated to play important roles in the immune defense in many fish species, especially in protection against pathogenic bacterial infection (Liu *et al*., 2016; Wang *et al*., 2016; Di Falco *et al*., 2017; Gou *et al*., 2018).

Both lysozymes genes are important biomarker molecules in evaluating immune and stress status in *C. gariepinus* aquaculture. However, to our knowledge, no reports have been documented on the molecular identification of these genes in *C. gariepinus* species. Moreover, the expression pattern of lysozyme genes after *A. hydrophila* challenges treatment are not documented yet. In the present study, we cloned partial cDNA of c-type and g-type lysozymes and evaluated their in vivo mRNA gene expressions after *A. hydrophila* infection. This study provides new information related to the immune genes of *C. gariepinus* species, and thus will extend our knowledge and provide a better understanding in *C. gariepinus* immune system.

MATERIAL AND METHODS

Primers design

Primers for cDNA amplification were designed based on the conserved sequences in the corresponding genes of several teleost fish available at gene database (GenBank; http://

Table 1. Primer used in this study

Name	Sequence (5'– 3')	Temperature (°C)	Application
LysC-F	GCTAAACGGTATGATCGGTGTGA	55	Partial identification
LysC-R	GCAGGAGATTTTACACCCGTT		
qCgLC F	CGGTATGATCGGTGTGAGCTGG	60	qPCR analysis
qCgLC R	CGGTTCTGGGCGTTGGTATTGA		
3'RACE Lc	AGTCGTACTTCAATACCAACGCCC	60	3' RACE PCR
LysG-F	GGCATTATATCCAGAGAGTC	56.5	Partial identification
LysG-R	CGAGCAACCACATCATTGG		
qCgLG F	CCTAACTGGCCCAAAGAGCA	60	qPCR analysis
qCgLG R	CCATACCCTCGTATGTGCGG		
3'RACE Lg	GAGGGGAGCATGGAACAGTAAGG		3' RACE PCR
Oligo(dT)$_{18}$	NNNN(T$_{18}$)	60	3' RACE PCR
Cg βactinF	ACCGGAGTCCATCACAATACCAGT	60	Internal control
Cg βactinR	GAGCTGCGTGTTGCCCCTGAG		

www.ncbi.nlm.nih.gov/genbank). The size of amplification product was predicted using primer-BLAST program (www.ncbi.nlm.nih.gov/tools/primer-blast). Melting temperature, dimers, and secondary structures were predicted using Oligo Analyzer 3.1 program (sg.idtdna.com/calc/analyzer). β-actin gene was used as an internal control and for expression level normalization. Real-time PCR primers were designed based on the results of the cloned sequences after partial identification. All primers used in this study were summarized at Table 1.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from the liver and head kidney using GENEzol™ reagent (Genaid, Taiwan) following the manufacturer's instruction. The concentration of total RNA was measured using spectrophotometer at optical densities of 260 and 280 nm, respectively. RNA purification and the first strand cDNA synthesis were carried out from 1 µg of total RNA using RevertraAce® qPCR RT Mastermix with gDNA remover kit (Toyobo, Japan) according to the manufacturer's manual. RNA integrity was evaluated by gel agarose electrophoresis method and gDNA contamination was verified by PCR-amplification using intron-spanning primer for β-actin genes. The cDNA was diluted in nucleases free water and stored at –20ºC prior to use.

Gene cloning

Partial sequences of *c-Lyz* and *g-Lyz* were amplified by PCR method. PCR reactions were performed in a total volume of 30 µL, consisting of 15 µL premix of MyTaq™ Red Mix (Bioline, UK), 1 µL (10 µM) of each primer, 12 µL nucleases free water, and 1 µL (50 ng/µL) of cDNA template from the fish liver. PCR thermal cycling programs were set as follow: 95°C for 3 min; 35 cycles of 95°C for 30 s, each primer annealing temperature for 30 s and 72°C for 30 second; and final extension at 72°C for 5 min. PCR products were separated on 1.5% agarose gel and purified using Wizard® SV Gel and PCR Clean-Up (Promega, USA) following kit protocol. Purified PCR products concentration was measured then ligated into pTA2 vector using Target Clone -Plus- (Toyobo, Japan). Ligated vector was transformed into *Escherichia coli* DH5α competent cells. Positive colonies were taken and verified by PCR. Plasmid isolation from positive colonies were performed using Illustra™ Plasmid Mini Kit (GE Healthcare, USA). Plasmid were amplified using universal T7 promotor and M13 reverse primers for sequencing. To obtain the 3'Untranslated Region (3'UTR) of the genes, the 3'RACE (rapid amplification of cDNA ends) was conducted using the 3'RACE primer that designed based on the cloned sequences.

Sequence reading and bioinformatic analysis

PCR product for sequencing was purified using ExoSAP-IT (GE Healthcare, USA). PCR labelling conducted using BigDye® Terminator v3. Cycle Sequencing Kit (Applied Biosystems, USA) and purified using BigDyeXTerminator™ Purification Kit (Applied Biosystems, USA). Sequence reading was performed using 3500 Genetic Analyzer machine (Applied Biosystems, USA). All sequencing preparation and reading was conducted in the Main Centre for Freshwater Aquaculture, Sukabumi, West-Java, Indonesia. Nucleotide sequence and its predicted amino acid residues were compared to other teleost and species using BLASTN and BLASTX program (blast.ncbi.nlm.nih.gov). Conserved domain of the genes was predicted using Conserved Domain Database (https://www.ncbi.nlm.nih.gov/cdd/) and SMART (http://smart.embl-heidelberg.de/) using the partial amino acid sequences. Phylogenetic tree was constructed using neighbor-joining method with default settings and 1000× bootstraps on MEGAX software (https://www.megasoftware.net) based on the partial amino acid sequences of each genes.

Bacterial infection

Pathogenic *A. hydrophila* was obtained from the Laboratory of Fish Health Management, IPB University, Indonesia, which was isolated from naturally infected fish at a local fish farm. The isolate was characterized using biochemical test. *A. hydrophila* was suspended in PBS solution at a final concentration of 10^7 CFU/mL, which was determined according to LD_{50} test prior to the experiment. Ninety individuals (60 ± 8 g) were acclimated at 28–29°C in 18 glass tanks (5 individuals/tank; 15 L) for one week. Fish were fed twice daily (08.00 and 17.00) with a commercial feed. Feed and fecal wastes were removed daily. Before injection, the fish was anesthetized with 100 mg/L MS222. Forty-five fish was injected with 0.1 mL of *A. hydrophila* suspension intra-muscularly as the treatment group, and forty-five other was injected by 0.1 mL PBS as a control group. Liver, head kidney, and spleen were collected from randomly selected fish at 0 h, 6 h, 12 h, 24 h, 48 h, and 96 h post-infection (hpi; n=3). Fish were deep-anesthetized with 200 mg/L MS222 for 2–3 min before tissues collection.

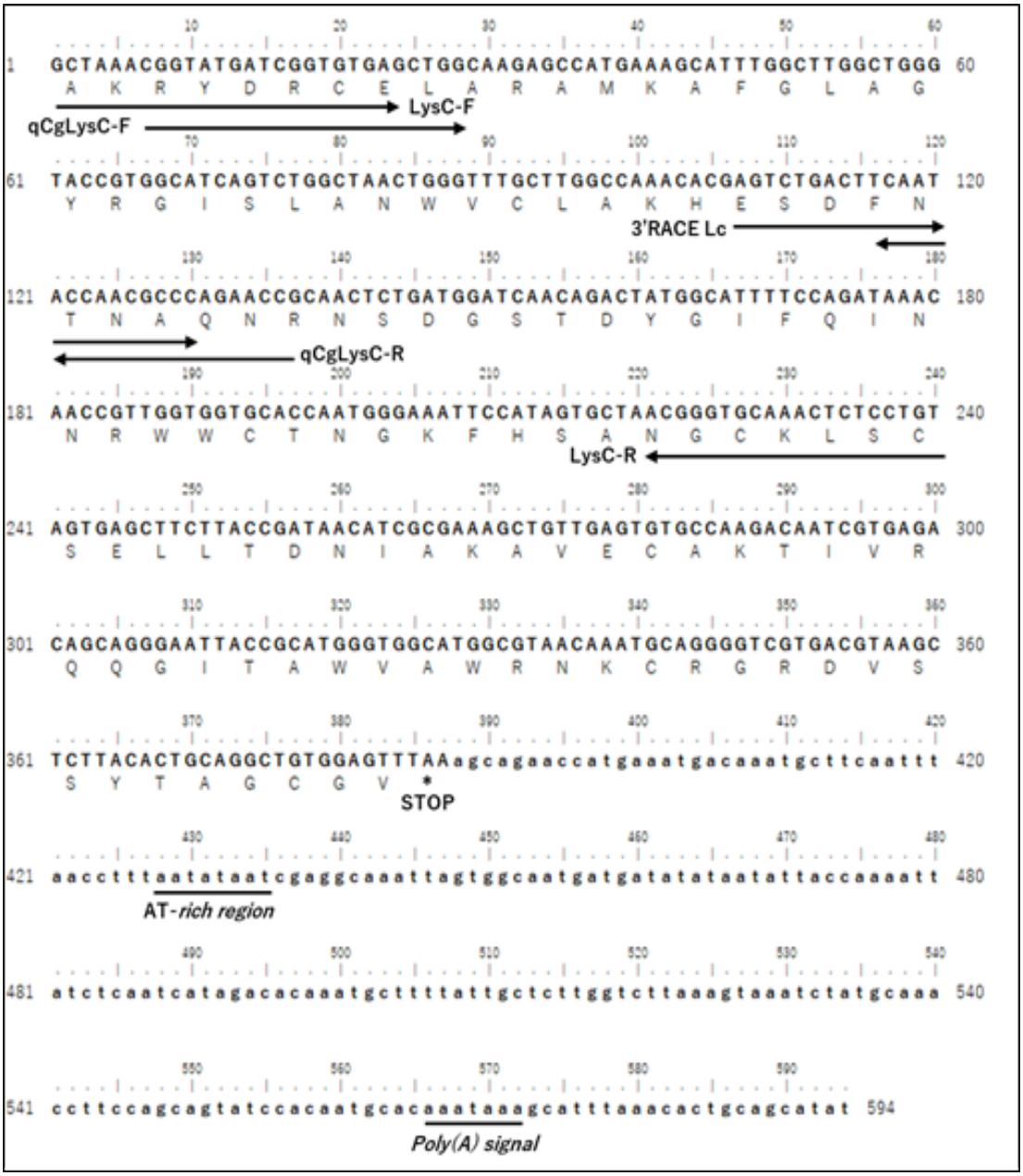


Figure 1. Partial nucleotide sequence of *C. gariepinus* c-Lyz gene

Gene expression and statistical analysis

Real-time PCR (qPCR) was used to determine mRNA expression of *c-Lyz* and *g-Lyz* genes. The qPCR reaction was performed in a Rotor-Gene 6000 (Corbett, USA) machine. An aliquot of 20 µL of total volume reaction containing 10 µL 2× SensiFAST SYBR® NO-ROX (Bioline, UK), 0.8 µL (10 µM) of each qPCR primer, 4 µL of 10 ngcDNA from the tissues, and 4.4 µL nucleases free water. The amplifying program was set at 95°C for 2 min, and 40 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 15 s. Melting curve analysis was conducted after amplification program to evaluate the primer specificity. Levels of all the genes were analyzed according to the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001) after normalized with the β-actin gene. The fold change of expression levels after bacterial infection were compared to the expression of the control group at the same time point. All expression data were presented as average ± SD. All statistical analyses were conducted in the SPSS v.17 software (SPSS Inc, USA).

RESULTS

Gene identification

The genes of *c-Lyz* and *g-Lyz* of *C. gariepinus* were successfully cloned and partially identified, which consisted of 594 and 560 of coding

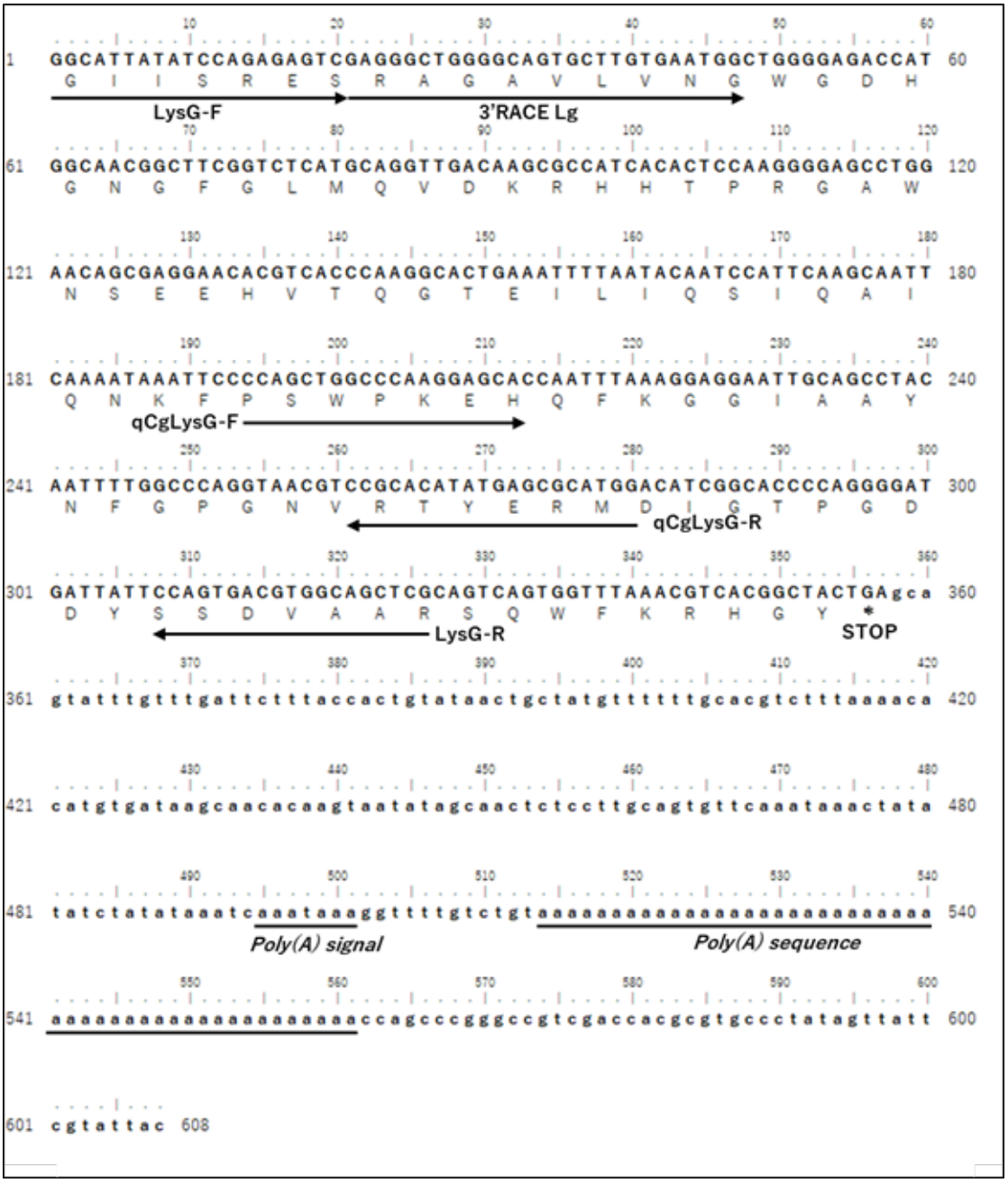


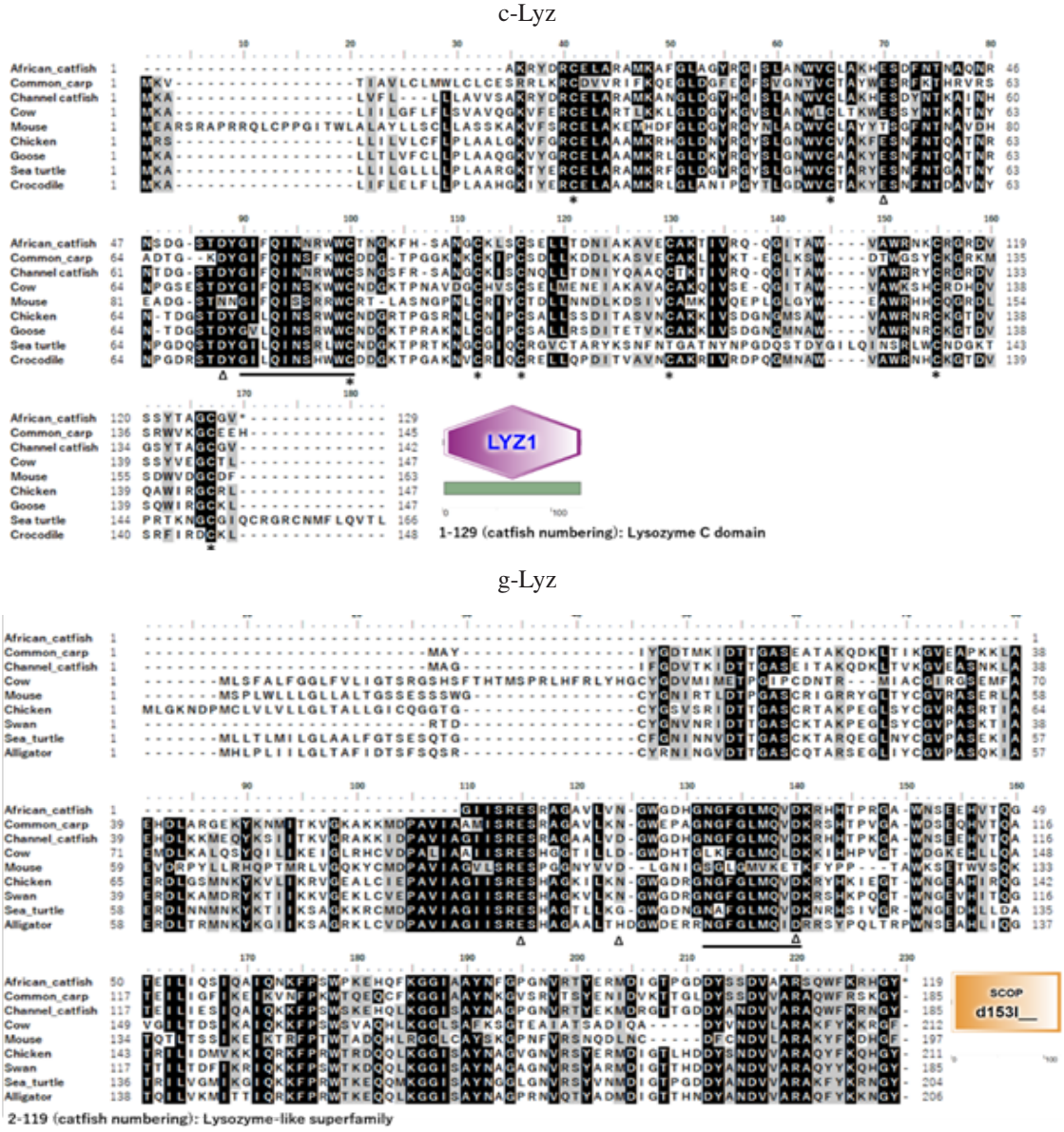
Figure 2. The partial nucleotide sequence of *C. gariepinus* g-Lyz gene

sequences, respectively. The corresponding mRNA sequences have been submitted to the GenBank under accession number of MK112877 (*c-Lyz*) and MH341528 (*g-Lyz*), respectively. The sequence features were presented in the Figure 1 and 2. Primers sequences were also marked within the gene sequences. The 3'UTR region of both gene were identified, including the AT-rich region and the poly-A sequence.

The translated amino acid residues for all the genes are showing high similarity with other teleost fish (Figure 3). All identified sequence belongf to lysozyme protein domain. Cysteine residues and catalytic sites of the enzymes, and the signature sequence of both lysozymes were also identified. The phylogenetic tree showed that all the identified genes were clustered in one clade with other fish species and closely related to other catfish species. Both *c-Lyz* and *g-Lyz* were separated from other group such as mammals, avian, and reptile (Figure 4).

Bacterial infection experiment

The mRNA levels of *c-Lyz* and *g-Lyz* were modulated after *A. hydrophila* injection with different patterns was observed for each organ (Figure 5). The *c-Lyz* expression was significantly modulated at 6 hpi ($P<0.01$ or $P<0.05$) in all



Figures 3. Multiple alignment of amino acid sequence of *C. gariepinus* lysozymes gene with other species. Identical residues are highlighted in black, grey shadows indicate similar residues within 75% similarity threshold, dashes (_) indicate gaps in the aligned amino acid sequences, asterisks * indicate cysteine residues, arrow heads Δ indicate catalytic sites. Signature sequences of the both lysozymes are underlined.

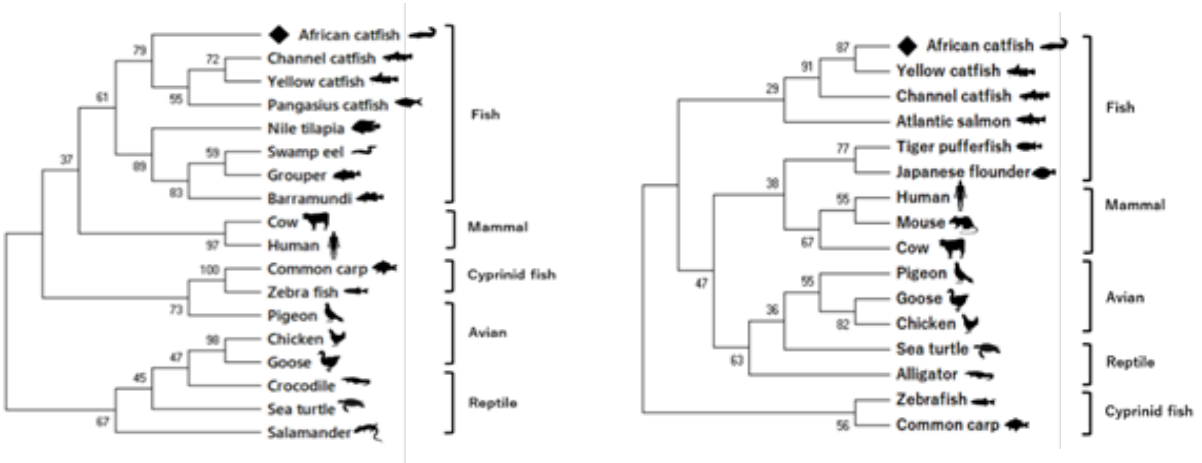


Figure 4. Phylogenetic tree of c-type and g-type lysozyme of African catfish and other species. Phylogenetic tree was constructed using neighbour-joining method with 1000 × bootstrap.

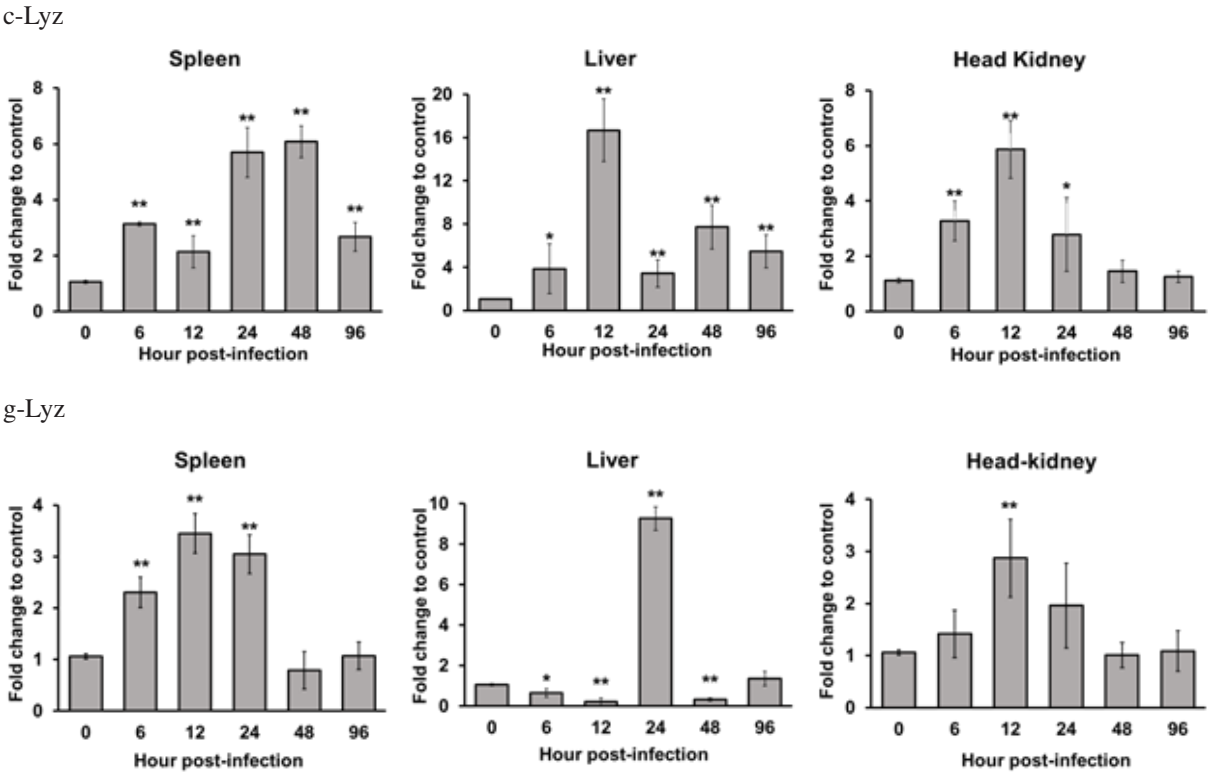


Figure 5. The expression pattern of lysozyme genes transcripts in the spleen, liver, and head kidney of *C. gariepinus* following injection with pathogenic *A. hydrophila*. Expression ratio was normalized to β-actin. Asterisks symbols indicate the significance in difference to the fish mRNA transcripts before the infection (*= p<0.05; **= p<0.01). Data was presented as mean ± SD (n= 3) and showed as fold change relative to the control group at the same time points.

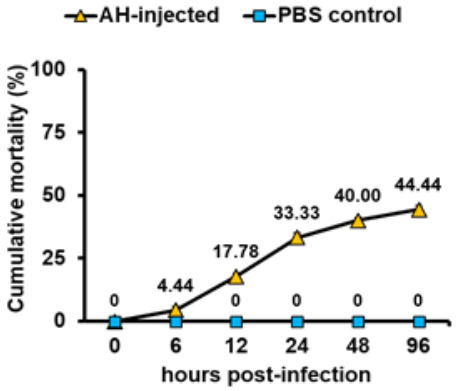


Figure 6. Cumulative mortality of *C. gariepinus* after subjected to 107 CFU/mL *A. hydrophila* injection

tissues, and maintained its significant expression until 96 hpi in spleen and liver (P<0.01 or P<0.05). Its expression in the head kidney was decreased to basal level at 48 and 96 hpi. The *g-Lyz* expression patterns in the spleen and head kidney were similar, it rapidly modulated at 6 hpi and reached a significant peak at 12 hpi (P<0.01), then decreased to its basal levels at 48 and 96 hpi. In the liver, *g-Lyz* expression was down-regulated at 6 and 12 hpi (P<0.05 or P<0.01) and after 12 hpi the expression levels reached its peak (P<0.01), thereafter it was down regulated to its basal levels.

The fish cumulative mortality following injection with *A. hydrophila* was also observed and presented in Figure 6. For the first 24 hpi, fish mortality in the injected group was about 33.33%. On the second day, fish cumulative mortality was 40% and became 44.44% at 96 hpi. No mortality was found at the control group during the experiment.

DISCUSSION

The innate immune system is a fundamental defense mechanism in fish and has some important roles in fish homeostasis and immune system

(Magnadóttir, 2006). One of its components, viz. lysozymes, act as the first line of defense in fish and provide the initial resistance to pathogens within the first hour of infection (Saurabh & Sahoo, 2008; Ye *et al*., 2010; Pridgeon *et al*., 2013; Zhang *et al*., 2018). The information related to the immune-related genes of African catfish is still scarce, and accordingly the immune response evaluation based on the immune genes on mRNA levels after bacterial infection and stressors induction is still limited. The lack of research on African catfish immune system remains as a major constraint in understanding the development of immune system, vaccine development, immune-stimulant and culture system evaluation, and selection of bacterial resistant strains through marked assisted selection or selective breeding. Considering the current situation on *C. gariepinus* immune research, it is necessary to enhance its research especially regarding to immune-related genes identification and its expression evaluation after bacterial challenge.

The present study provides the sequence information and the mRNA levels evaluation of African catfish *c-Lyz* and *g-Lyz* genes after *A. hydrophila* infection. Based on the partial identification results, the amino acid residues of the genes were similar to the corresponding sequences in other teleost fishes (Figure 1). African catfish c-type and g-type lysozymes possessed two and three catalytic sites and showed homology with other fish species (Liu *et al*., 2016; Zhang *et al*., 2018). The signature sequences of *c-Lyz* and *g-Lyz* and the conserved cysteine residues was also found within the *C. gariepinus* sequences, which was also reported in most of the fish species (Buonocore *et al*., 2014; Wohlkönig *et al*., 2010). These results indicated that the cloned and identified genes are the lysozyme genes of African catfish. However, full-length characterization is still needed to extend our knowledge about the genes function in immune and stress responses of *C. gariepinus*.

Rapid induction was shown by all the genes in all examined tissues after bacterial infection with different patterns were observed in different organs tested in this study. This indicates that their important roles as immune components for early defense system against bacterial infection (Figure 5). Chicken type and goose type lysozyme have a relatively different patterns of induction in the liver. The *c-Lyz* expression was induced in the early time of infection while *g-Lyz* expression was decreased in the same time points in the liver. After 24 h they 'shifted' their expression. Such expression pattern also reported in the liver of Darby's sturgeon *Acipenser dabryanus* (Zhang *et al*., 2018) and channel catfish *Ictalurus punctatus* (Wang *et al*., 2013) after *A. hydrophila* and *Edwardsiella ictaluri* infection. This pattern was suggested as "collaborative" functions of the lysozymes after bacterial infection (Wang *et al*., 2013). Up-regulation of both lysozyme mRNA expressions after bacterial challenge also widely reported in teleost fish. In grass carp *Ctenopharyngodon idella*, g-type lysozyme expression was significantly up-regulated after 24 h, 72 h and 7 days in the liver, head kidney, and spleen after *A. hydrophila* injection, but only after 72 h in gill for c-type lysozyme (Ye *et al*., 2010). The c-type and g-type lysozyme of *I. puncatatus* were also significantly increased in the liver, spleen, and head-kidney after *E. tarda* injection (Wang *et al*., 2013). In general, after bacterial infection in *C. gariepinus, c-Lyz* mRNA expressions were induced higher than *g-Lyz*. This result suggest that both lysozymes may play a crucial role in the immunological responses of African catfish against *A. hydrophila* infection.

CONCLUSION

We have identified and cloned the partial cDNA of c-type lysozyme and g-type lysozyme from African catfish *C. gariepinus*. Their expression modulation in immune tissues after *A. hydrophila* injection marked their importance in host immune response against bacterial infection. This study provided valuable information that can be used to understand the African catfish immune systems and to provide better disease and stress management in *C. gariepinus* culture. However, further studies to fully-characterize the corresponding genes will extend our knowledge about its molecular function and mechanisms.

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