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Expression of miR-29, miR-125, and miR-181 in the anterior kidneys of Streptococcus-infected Nile tilapia (*Oreochromis niloticus*)

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Abstract

MicroRNAs (miRNAs) are a group of small non-coding RNAs which posttranscriptionally regulate gene expression in eukaryotic cells. In this study, three miRNAs participating in the immune function in mammalian cells: miR-29, miR-125, and miR-181 have been characterized in Nile tilapia (*Oreochromis niloticus*). A comparison of all three miRNAs in Nile tilapia and other organisms, including zebrafish, mouse, rat, chicken, pig, and human revealed identical sequence of mature miRNA sequences. The genomic sequences of miR-29, miR-125, and miR-181 of Nile tilapia have been amplified using polymerase chain reaction (PCR) and cloned into pGT-19T plasmid. The plasmids containing the sequences of miR-29, miR-125, and miR-181 were used for standardization and validation of quantitative PCR (qPCR) methods. Moreover, the qPCR methods have been applied as a tool to determine the expression of all three miRNAs in the anterior kidneys of *Streptococcus agalactiae*-infected tilapia. Specifically, expression of miR-29, miR-125, and miR-181 reduced significantly at 3 days post bacterial challenge. *In silico* analysis of three miRNA targets revealed that multiple genes could be regulated by these miRNAs including *Ifi30*, *Cd276*, *Lfng*, *Cdc42se1*, *Ddx3x*, *Il1a*, and *Atg5*. In conclusion, these results demonstrated the expression level and molecular targets of miRNAs in Nile tilapia which could be applied for the understanding of these miRNAs during pathogen-host interaction.

Key Words: microRNAs, Nile tilapia, qPCR, *Streptococcus agalactiae*

Introduction

Tilapia is the second most important freshwater aquaculture species that are cultured worldwide[34)]. Although genetic improvements and aquaculture technologies have been implemented for tilapia

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production, the intensive farming system and fish rearing in the open environment make them more susceptible to various diseases. Among these, Streptococcus infection by *Streptococcus agalactiae* is one of the most important infectious diseases in tilapia[20]. The clinical signs of infection include skin darkening, corneal opacity, exophthalmia, and erratic swimming due to the damage of the central nervous system[31]. Previously, two species of Streptococcus: *S. agalactiae* and *S. iniae* have been isolated from moribund tilapia in Thailand[21]. The cumulative mortality of affected farms may reach 20–30% at the end of harvest. To reduce the economic impact of Streptococcus infection, antibiotics and vaccines have been implemented for disease control. Nevertheless, the complete understanding of molecular mechanisms during Streptococcus infection may lead to better intervention strategies or designing new therapeutic products. One of the potential approaches is to regulate the host immune response by manipulating miRNA functions, as this concept had been shown to be effective in tilapia and other animals[42,53].

In teleost fish, the immune system plays essential roles in controlling of various infectious diseases. Among the lymphoid tissues of fish, the anterior kidney is one of the most important hemopoietic tissues for the production of red and white blood cells[1]. Moreover, multiple blood-borne pathogens are quickly removed from the circulation in the specialized capillaries in the anterior kidney[44]. MicroRNAs (miRNAs) are a group of small non-coding, 20–22 nucleotides long RNAs that regulate host gene expression through complementary binding to target messenger RNA (mRNA)[5]. In 1993, a small RNA, lin-4, which regulates lin-14 protein, was first discovered in *Caenorhabditis elegans*[29]. Subsequent studies then indicated that miRNAs are endogenously expressed in insects, plants, and animals[3,11,32]. The biogenesis of miRNAs starts with the cleavage of pri-miRNAs into pre-miRNAs by an RNase-III enzyme, Drosha, in the nucleus. The pre-miRNAs are, thereby, transported into cytoplasm for further process into mature miRNAs[16]. In mammalian cells, a set of miRNAs, including miR-29, miR-125, and miR-181 had been shown to regulate immune responses. Previous studies described the role of miR-29 during T cell development in the thymus and its function during bacterial infection[33,49]. In addition, an overexpression of miR-125 in the bone marrow-derived macrophages inhibits tumor progression in C57BL/6 mice by directly targeting interferon regulatory factor (IRF4)[6]. Moreover, recent studies showed that miR-181 controls natural killer (NK) cell development[7] and T cell response to antigen peptide stimulation[17,30]. Like mammals, a number of studies suggested that miRNAs participated in physiological and pathological responses in teleost fish, for instance, 270 of miRNAs were identified using a custom microarray analysis[8]. Moreover, analysis of grouper (*Epinephelus coioides*) spleen revealed that 45 grouper miRNAs upregulated more than 1.5-fold during Singapore grouper iridovirus infection[15]. To predict the target of miRNAs in organisms, computer programs and web-based tools, such as miRBase has been applied. In the miRBase, a number of miRNA sequences derived from various fish species, including carp (*Cyprinus carpio*), zebrafish (*Danio rerio*), pufferfish (*Fugu rubripes*), and salmon (*Salmo salar*) are available for sequence comparison. However, the molecular mechanisms and target analysis of immune-related miRNAs have not been comprehensively investigated in Nile tilapia.

The objective of this study is to develop a quantitative PCR (qPCR) method for the determination of the expression levels of three miRNAs, including miR-29, miR-125, and miR-181 in anterior kidneys of Nile tilapia. In addition, this newly developed qPCR protocol has been applied to characterize the expression of miRNAs during *S. agalactiae* infection.

Materials and Methods

Fish and general management: A sample of 40 Nile tilapia (*Oreochromis niloticus*), with an average weight of 30 g, were obtained from a local fish farm in Saraburi province, Thailand. Fish were acclimatized in 400 l plastic tanks for 1 week with 100% water change on a daily basis. The water quality parameters including dissolved oxygen, temperature, ammonia, and nitrite were daily monitored. Fish were fed with tilapia commercial diet twice a day at 3% body weight. The fish were handled according to the guidelines and regulations of the Kasetsart University animal care and use guideline, and animal use permit was approved by the Kasetsart University animal use committee (IACUC number OACKU00659).

Tissue collection: In order to acquire fish tissues for further investigation, 3 Nile tilapia were euthanized in eugenol solution (Aquanes, Better Pharma, Thailand) at 2 ml/ 1 l water for 10 min. The signs of euthanization, including stopped operculum and body movement for at least 10 min, were monitored prior to necropsy examination. The anterior kidneys (100 mg) from individual fish were aseptically collected and transferred into 1.5 ml centrifuge tubes containing 1 ml TRIzol™ reagent (Life Technologies, California, USA).

RNA isolation and cDNA synthesis: The anterior kidney tissue was homogenized in TRIzol™ reagent and further processed for RNA isolation according to the manufacturer's protocols. The quantity and quality of the RNAs were determined using Nanodrop spectrophotometer (Nanodrop 2000, ThermoScientific, CA, USA). Samples with 260/280 absorbance ratio higher than 1.80 are considered high purity, and therefore will be processed for further PCR reactions. Five µl of RNA was reversely transcribed to complementary DNAs (cDNAs) using Viva 2-steps RT-PCR kit (Vivantis technologies, Selangor Darul Ehsan, Malaysia) according to the manufacturer's instructions.

Sequence analysis and primer design: The mature sequences of miR-29, miR-125, and miR-181 of other organisms, including zebrafish (*Danio rerio*), human (*Homo sapiens*), mouse (*Mus musculus*), rat (*Rattus norvegicus*), pig (*Sus scrofa*), and domestic chicken (*Gallus gallus*) were retrieved from the miRBase database (http://mirbase.org)[12–14,26,27] (Table 1). The nucleotide sequences of miR-29, miR-125, and miR-181 were blasted to Nile tilapia genome sequences (Acc. No. XR_001224982, XR_267768, and XR_270030) using MAFFT multiple sequence alignment program (http://mafft.cbrc.jp/alignment/server/). Subsequently, the forward and reverse primers of each tilapia miRNA was designed using Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). As the mature miRNAs are quite small (20–24 bp), the qPCR was designed from pre-miRNA sequences (80 to 180 bp) which covered the entire sequence of mature miRNA. The forward and reverse primers of internal control gene, actin beta (*Actb*), was obtained from a previous publication[48] (Table 2).

Plasmid construction: The specific PCR products were amplified from cDNA isolated from anterior kidney of healthy Nile tilapia. The polymerase chain reaction (PCR) technique was carried out in a T100 thermal cycler (Bio-Rad, California, USA) following this cycle: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 1 min, and final elongation at 72°C for 5 min. The size of the PCR products were separated on agarose gel and cloned into pTG19-T vector (Vivantis Technologies, Selangor Darul Ehsan, Malaysia) using a T4 DNA ligase enzyme (New England BioLabs, Massachusetts, USA). The plasmid containing tilapia miRNAs were transformed into competent *Escherichia coli* strain DH5alpha to use as a template for qPCR validation. The blue/white colonies were screened

Table 1. Comparison of mature miRNAs sequences of tilapia and other organisms

miRNA name	Species	miRNA mature sequence	Length (mer)	miRBase accession number
dre-miR-29a	*Danio rerio*	UAGCACCAUUUGAAAUCGGUUA	22	MIMAT0001802
hsa-miR-29a-3p	*Homo sapiens*	UAGCACCAUCUGAAAUCGGUUA	22	MIMAT0000086
mmu-miR-29a-3p	*Mus musculus*	UAGCACCAUCUGAAAUCGGUUA	22	MIMAT0000535
rno-miR-29a-3p	*Rattus norvegicus*	UAGCACCAUCUGAAAUCGGUUA	22	MIMAT0000802
ssc-miR-29a	*Sus scrofa*	CUAGCACCAUCUGAAAUCGGUUA	23	MIMAT0013870
gga-miR-29a-3p	*Gallus gallus*	UAGCACCAUUUGAAAUCGGUU	21	MIMAT0001096
oni-miR-29	*Oreochromis niloticus*	UAGCACCAUUUGAAAUCGGUUA	22	–
dre-miR-125a	*Danio rerio*	UCCCUGAGACCCUUAACCUGUG	22	MIMAT0001820
hsa-miR-125a-5p	*Homo sapiens*	UCCCUGAGACCCUUUAACCUGUGA	24	MIMAT0000443
mmu-miR-125a-5p	*Mus musculus*	UCCCUGAGACCCUUUAACCUGUGA	24	MIMAT0000135
rno-miR-125a-5p	*Rattus norvegicus*	UCCCUGAGACCCUUUAACCUGUGA	24	MIMAT0000829
ssc-miR-125a	*Sus scrofa*	UCCCUGAGACCCUUUAACCUGUG	23	MIMAT0013897
oni-miR-125	*Oreochromis niloticus*	UCCCUGAGACCCUUAACCUGUGA	23	–
dre-miR-181a-5p	*Danio rerio*	AACAUUCAACGCUGUCGGUGAGU	23	MIMAT0001623
hsa-miR-181a-5p	*Homo sapiens*	AACAUUCAACGCUGUCGGUGAGU	23	MIMAT0000256
mmu-miR-181a-5p	*Mus musculus*	AACAUUCAACGCUGUCGGUGAGU	23	MIMAT0000210
rno-miR-181a-5p	*Rattus norvegicus*	AACAUUCAACGCUGUCGGUGAGU	23	MIMAT0000858
ssc-miR-181a	*Sus scrofa*	AACAUUCAACGCUGUCGGUGAGUU	24	MIMAT0010191
gga-miR-181a-5p	*Gallus gallus*	AACAUUCAACGCUGUCGGUGAGU	23	MIMAT0001168
oni-miR-181	*Oreochromis niloticus*	AACAUUCAACGCUGUCGGUGAGU	23	–

and PCR amplified in a T100 thermal cycler (Bio-Rad, CA, USA) using specific primers for each miRNA. The recombinant plasmid containing miRNA fragments were then purified using Presto mini plasmid kit (Geneaid, New Taipei City, Taiwan) and submitted for nucleotide sequencing (Macrogen, Seoul, Korea).

Real-time PCR assay: The plasmids containing tilapia miR-29, miR-125, and miR-181 were used for qPCR standardization. Briefly, each plasmid was ten-fold serial diluted from 10^{-2} to 10^{-6} of the 1 ng stock concentration for real-time amplification. One ng of miR-29, miR-125, and miR-181 plasmid DNA was equal to 3.08×10^9, 3.11×10^9, and 3.09×10^9 copy numbers, respectively. The conversion of plasmid DNA concentration into an amount of copies was calculated using the following formula: Copy number = ([Amount of DNA (ng)] $\times$ 6.022×10^{23}) / ([Template length (bp)] $\times$ $1 \times 10^9 \times 650$), where 1×10^9 refers to the conversion into ng, and 650 refers to the average weight of a base pair. Since miRNA sequences were inserted into plasmid pTG19-T cloning vector which had a size of 2,880 bp, the total lengths of plasmid DNA of miR-29, miR-125, and miR-181 were equal to 3,005, 2,978, and 3,000 bp, respectively. The qPCR reactions were performed in a 20 μl reaction containing 10 μl 2x SYBR mastermix (BioRad, CA, USA), 0.8 mM of forward and reverse primers each, 4 μl of plasmid template, and 4.4 μl of molecular water to adjust the final volume. Each dilution was run in triplicate to allow the reproducibility of the mean Ct values. The amplification and melting curves were determined in a CFX96 real-time PCR thermocycler (BioRad, CA, USA). To verify the size of the PCR products, samples were separated on a low melting agarose gel (Nusieve 3 : 1, Lonza, Switzerland). Five μl of each PCR

Table 2. Sequences of primers used in this study

Primer name	Sequence (5'-3')	Length (mer)	Product size (bp)
miR-29 Forward	GTCTACAGTGACGTTTTGCCAG	22	125
miR-29 Reverse	CTGTCCTTCACTGTAACCGAT	21	
miR-125 Forward	CAACTGGCTCCGTCTTTACAC	21	98
miR-125 Reverse	GTGACCTGCTTTGACCTCAC	20	
miR-181 Forward	TTGCTTGCCTCGGTGAACATT	21	120
miR-181 Reverse	TCTTCCTTCGCTCGTTCCTGA	21	
Actb Forward*	TCCAATTTATTGGCCTTCGTTGC	23	114
Actb Reverse*	CTTCCATTTTCTGTGTGAGGGAGG	24	

*Primer sequences obtained from[48)]

product was mixed with 1 μl of 6x orange DNA loading dye (Thermo Scientific, Massachusetts, USA). After gel electrophoresis, samples were stained with ethidium bromide, and analyzed using a Gel Doc EZ Gel Documentation System (Bio-Rad, California, USA).

Expression of miRNAs in S. agalactiae-infected Nile tilapia: The bacteria, *S. agalactiae* strain VET-KU01, were isolated from clinically sick tilapia in Thailand. The bacteria were cultured on tryptic soy broth (TSB) for 24 hr. The bacterial suspension was centrifuged at 2,500 ×g for 15 min to collect bacterial pellets. The pellets were resuspended in 1 ml phosphate buffered saline (PBS) and adjusted to a final concentration of 10^8 CFU/ml. The bacterial concentration was equal to OD 0.6 as determined by using BlueStar series UV-Vis spectrophotometer (LabTech, Massachusetts, USA). As for the challenge experiment, 20 fish were intraperitoneally injected with 100 μl of 10^8 CFU/ml bacterial suspension, meanwhile the control group was intraperitoneally injected with 100 μl PBS to use as a non-challenge control group. The clinical signs of streptococcal infection, which include the stoppage of feeding, exophthalmia, multiple abscesses on the skin, and erratic swimming, were monitored daily until the end of the experiment. At 24, 48, 72, and 96 hr after bacterial challenge, the anterior kidneys were collected from 5 individual fish at each time points for RNA isolation. RNA extraction, cDNA synthesis, and quantitative amplification were conducted as described. The mean Ct values of each miRNAs were normalized to the mean Ct values of *Actb* genes. The $2^{(-\Delta\Delta Ct)}$ values were calculated and compared between the *S. agalactiae* challenge group and the non-challenge control group.

In silico identification of miRNA target genes: The miRNA target genes were identified by using two online miRNA target prediction programs; DIANA microT-CDS (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT_CDS/index/)[39,43)], and miRmap (http://mirmap.ezlab.org/app/)[45)]. As the databases of both prediction programs do not contain tilapia miRNAs, the target genes were extrapolated from *Mus musculus* miRNAs. The highest scores as determined by DIANA microT-CDS (1.000 miTG score) or miRmap program (100.00 miRmap score) were selected and then screened for immune associated functions using KEGG database (http://www.genome.jp/kegg/kegg1.html/)[23-25)].

Statistical analysis: The difference of miRNA expression levels between the control and bacterial infected groups were analyzed with student's *t*-test. A p value of < 0.05 was considered statistically significant.

Results

Development and validation of qPCR for miRNA detection

The sequences of mature miRNAs of miR-29, miR-125, and miR-181 from zebrafish (*Danio rerio*), human (*Homo sapiens*), mouse (*Mus musculus*), rat (*Rattus norvegicus*), pig (*Sus scrofa*), and domestic chicken (*Gallus gallus*) were retrieved from the NCBI database and blasted against Nile tilapia genome to unravel similar sequences. Notably, the mature sequences of all three miRNAs of Nile tilapia shared high sequence identity to miRNAs from other organisms (Table 1). Subsequently, mature miRNAs of miR-29, miR-125, and miR-181 were amplified from tilapia anterior kidneys, and processed for qPCR validation. Each plasmid was 10-fold serial diluted from 10^{-2} to 10^{-6} for qPCR amplification. As shown in Fig. 1, the amplification curves of miR-29, miR-125, and miR-181 demonstrated mean Ct values ranging from 10 to 26 cycles. The slopes of the standard curves of miR-29, miR-125, and miR-181 were −3.394, −3.370, and −3.625, and the coefficient of determination (R^2) were 0.9998, 0.9995, and 0.9997, respectively. The calculated amplification efficiency of miR-29, miR-125, and miR-181 qPCR protocols were 97.08%, 98.03%, and 88.74%, respectively (Fig. 1E, F). No amplification curves were observed in the no template control samples of all qPCR reactions (data not shown). The calculation of standard deviation (SD) and coefficient of variance (CV) values of all three miRNAs were performed to assess the reproducibility of the real-time PCR protocol. The SD values acquired from miR-29, miR-125, and miR-181 ranged from 0.03 to 0.15, 0.06 to 0.22, and 0.06 to 0.14 respectively. The CV values were in range of 0.19% to 0.82%, 0.25% to 1.19%, and 0.24% to 0.99% for miR-29, miR-125, and miR-181, respectively.

Specificity of qPCR assay

The specificity of qPCR assays was determined using melting curve analysis. The melting curve analysis revealed a single melt peak from 78°C to 82°C (Fig. 2). In particular, the melting temperature (Tm) of PCR products amplified from miR-29, miR-125, and miR-181 primers were 78–78.5°C, 77–78°C, and 82–82.5°C, respectively. Furthermore, the specificity of qPCR assays was confirmed by the separation of PCR products on agarose gel. Single bands with the size of 125 bp, 98 bp, 120 bp, and 114 bp were observed for miR-29, miR-125, miR-181, and actin beta (*Actb*) respectively (Fig. 3).

Expression of miRNAs in S. agalactiae infection

The newly developed qPCR protocols have been applied to characterize the expression of miR-29, miR-125, and miR-181 in *S. agalactiae*-challenged tilapia. All challenged fish developed clinical signs of Streptococcus infection including bilateral exophthalmia, erratic swimming, and multiple abscesses at the fin and the head. The infected fish were confirmed positive for *S. agalactiae* using bacterial isolation on tryptic soy agar (TSA). Notably, at 72 hr post-challenge, the expression of miR-29, miR-125, and miR-181 in *S. agalactiae*-infected tilapia decreased significantly compared to the control fish. At 48 hr post-challenge, only miR-29 expression in infected fish significantly decreased. Lastly, the level of miR-181 in infected tilapia increased at 24 hr, but not significantly, then gradually declined in the following time-points (Fig. 4).

The miRNA target genes as predicted by two online web-based programs; DIANA microT-CDS program and miRmap program revealed that multiple immune associated genes including *Ifi30* and *Cd276* were targeted by miR-29. Similarly, the predicted target genes of miR-125 are *Lfng* and *Cdc42se1*, while the predicted target genes of miR-181 are *Ddx3x*, *Il1a*, and *Atg5* (Table 3).

Discussion

MiRNAs are small non-coding RNAs that

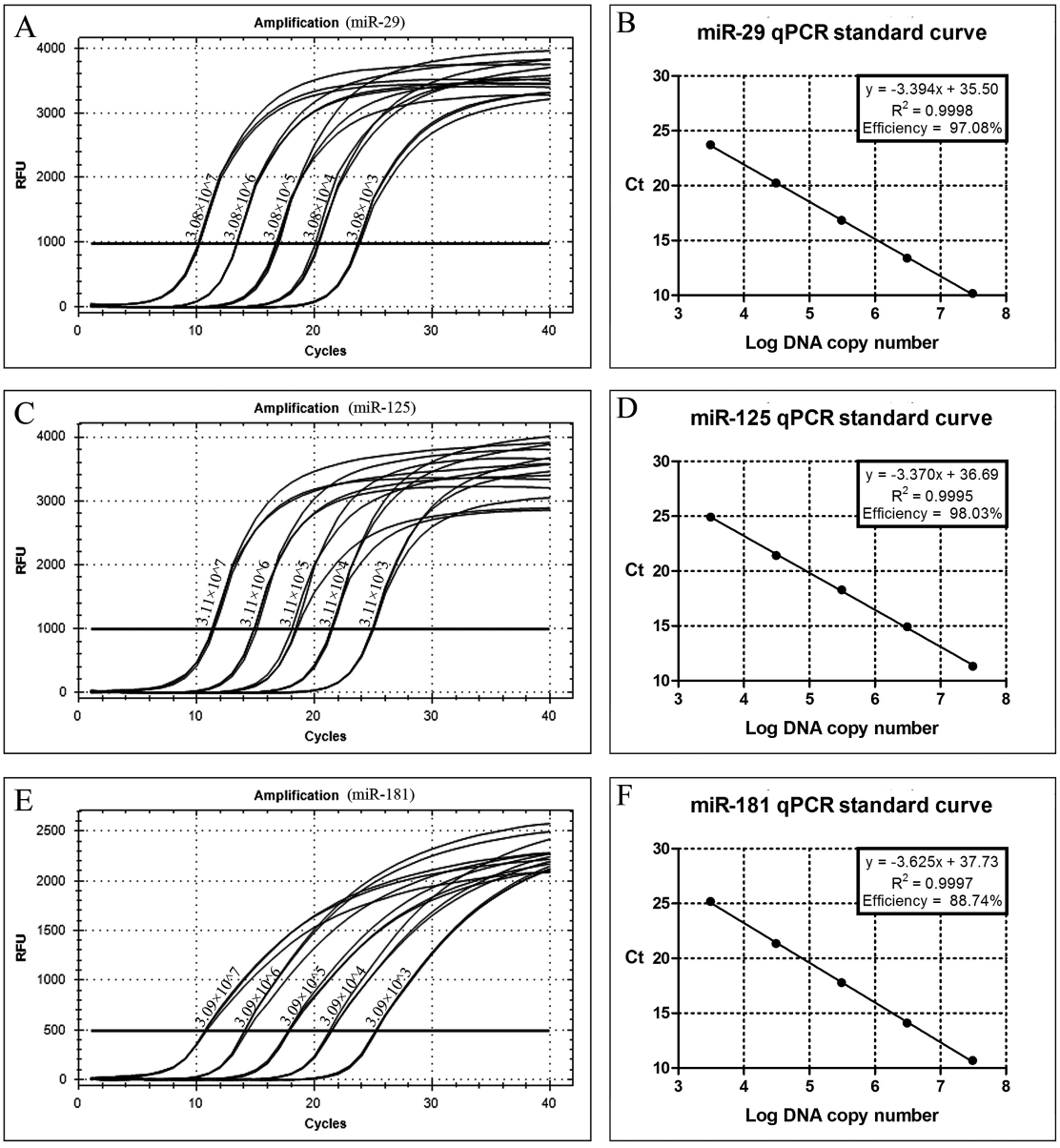

Fig. 1. Amplification curve of three tilapia miRNAs and standard curve analysis of qPCR reactions. The plasmids containing miR-29, miR-125, and miR-181 are 10-fold serial diluted ranging from 10^3 to 10^7 copy numbers and used as templates for qPCR optimization. The amplification curves are demonstrated for each miRNAs; miR-29 (A), miR-125 (C), and miR-181 (E). The standard curves are plotted from mean Ct values of each serial dilution of miR-29 (B), miR-125 (D), and miR-181 (F).

regulate host gene expression posttranscriptionally through complementary binding of target mRNA, leading to mRNA degradation or inhibition of protein translation. To date, more than one thousand miRNAs have been identified in insects, teleost fish, plants, and mammals[47,50–52]. Although extensive studies of miRNAs have been reported in other organisms, few reports on the roles of miRNAs and their expression levels have been characterized in Nile tilapia. Similar to mammals, the immune system of fish protects them from various pathogens. Fish has hematopoietic and lymphoid tissues, such as the anterior kidney that plays essential roles during pathogen clearance. Understanding the molecular functions of cells in this organ could provide

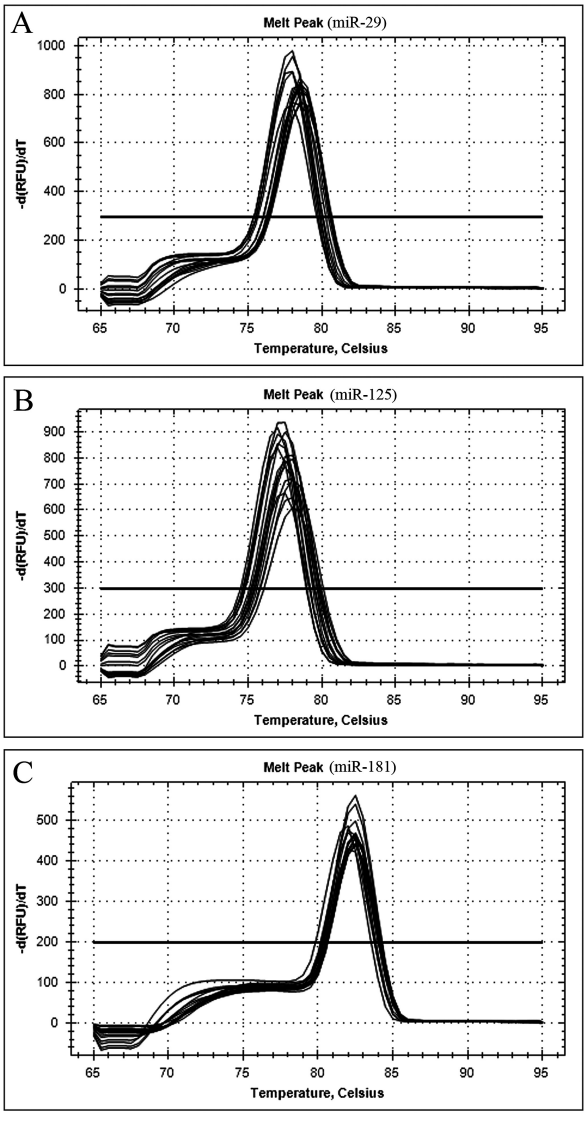

Fig. 2. Melting curve analysis of three tilapia miRNAs. The melting temperatures for miR-29 (A), miR-125 (B), and miR-181 (C) are 78–78.5°C, 77–78°C, and 82–82.5°C, respectively.

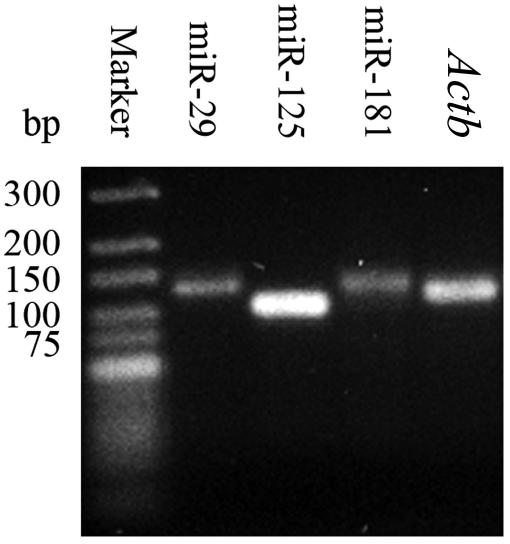

Fig. 3. PCR products of three tilapia miRNAs. RNAs were collected from the anterior kidneys of three individual normal fish. The PCR products were separated on low melting agarose gel (5% NuSieve agarose gel) showing a specific band size of 125 bp (miR-29), 98 bp (miR-125), 120 bp (miR-181), and 114 bp (*Actb*; internal control gene).

important information how the immune system is regulated in fish. In this study, a sensitive, specific, and reproducible qPCR protocol have been developed to quantify the expression of miR-29, miR-125, and miR-181 in normal and bacteria-challenged tilapia. Specifically, the standard curve analysis demonstrates the sensitivity of the qPCR protocol, which is able to detect all three miRNAs within the range of 10^3 to 10^7 miRNA copies. The slope, R^2, and amplification efficiency of the developed qPCR protocols are in the range of other works[37,38], indicating high sensitivity and accuracy of the developed qPCR protocols. All the mean CV values are below 5% variation, suggesting the reproducibility of the assays[2]. Moreover, the specificity of these protocols was evaluated from the melting curve analysis and the separation of qPCR products on agarose gel. In particular, single bands of 125, 98, and 120 bp which are equivalent to the expected sizes of tilapia miR-29, miR-125, and miR-181 were revealed on an agarose gel, suggesting the specificity of the developed qPCR protocol. Similar to this study, qPCR protocols have been developed to determine other miRNAs expression in tilapia tissues. For example, One study had characterized miRNA expression in skeletal muscle of tilapia using qPCR and next-generation sequencing[18]. Moreover, a set of 14 miRNAs were significantly altered at 6 to 48 hr post *S. agalactiae* infection in tilapia[46].

In this study, the results suggested that miR-29, miR-125, and miR-181 was downregulated in the presence of *S. agalactiae* infection, in

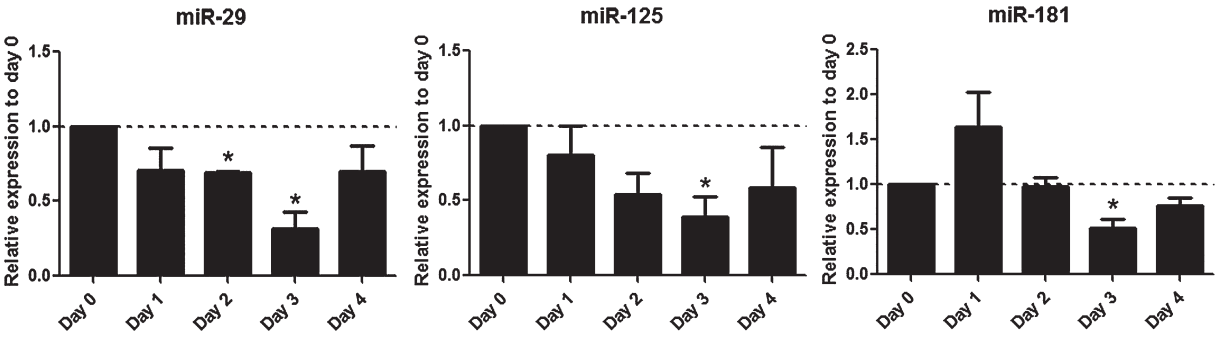

Fig. 4. Expression of three miRNAs in Streptococcus-infected tilapia. Fish were intraperitoneally injected with *S. agalactiae* at 10^8 CFU/ml. RNAs (n = 5 in each time point) were isolated from the anterior kidneys of normal and infected fish at 24, 48, 72, and 96 hr post-challenge. Expressions of miR-29, miR-125, and miR-181 were calculated and normalized to an internal control gene, *Actb*, using the $2^{(-\Delta\Delta Ct)}$ calculation method. *Significantly different expression levels relative to day 0 ($P < 0.05$).

Table 3. Prediction of three miRNA targets; miR-29, miR-125, and miR-181 using two miRNA target prediction programs; DIANA microT-CDS and miRmap

Rank	mmu-miR-29a-3p				mmu-miR-125a-5p				mmu-miR-181a-5p			
	DIANA microT-CDS		miRmap		DIANA microT-CDS		miRmap		DIANA microT-CDS		miRmap	
	Target gene	miTG score	Target gene	miRmap score	Target gene	miTG score	Target gene	miRmap score	Target gene	miTG score	Target gene	miRmap score
1	*Ddx3x*	1.000	*Rhoh*	99.47	*Sema4d*	1.000	*Vsir*	99.67	*Ddx3x**	0.998	*Il1a**	98.90
2	*Igf1*	1.000	*Traf3*	99.41	*Irf4*	1.000	*Arrb1*	99.53	*Il1a**	0.997	*Rora*	98.77
3	*Ifi30**	0.999	*Tnfrsf26*	99.06	*Tnfsf4*	1.000	*Treml2*	99.26	*Atg5**	0.993	*Ddx3x**	98.21
4	*Cd276**	0.995	*Atrn*	98.35	*Lfng**	1.000	*Lfng**	99.07	*Rps6ka3*	0.991	*Cd2ap*	98.12
5	*Icos*	0.994	*Cd244*	98.25	*Bak1*	0.999	*Ifih1*	98.80	*Nfat5*	0.980	*Atg5**	98.00
6	*Fer*	0.994	*Ifi30**	97.95	*Map4k2*	0.994	*Traf6*	98.79	*Lif*	0.979	*Chst2*	97.90
7	*Tfeb*	0.986	*Cd276**	97.74	*Cd34*	0.988	*Sarm1*	98.71	*Braf*	0.979	*Tnfsf10*	97.86
8	*Mafb*	0.982	*Eomes*	97.68	*Rora*	0.987	*Stk10*	98.67	*Foxp1*	0.976	*Cxadr*	96.73
9	*Tnfrsf1a*	0.980	*Nfat5*	97.67	*Cdc42se1**	0.987	*Lif*	98.61	*Plcl2*	0.972	*Ereg*	96.38
10	*Bcl11a*	0.973	*Il21*	97.66	*Tnfaip3*	0.980	*Cdc42se1**	98.58	*Tnfrsf11b*	0.968	*Rhoh*	95.75

*Target genes of each miRNA that received high prediction scores from both programs.

particular, during early bacterial infection at 2–3 days post-infection. It has been shown that innate immune-associated genes are regulated through various miRNAs[4,22]. Thus, the downregulation of miRNAs which suppresses immune effector genes may allow these target genes to translate to proteins that support bacterial clearance. In fact, recent studies in tilapia indicated that an upregulation of three miRNAs: miR-310-3p, miR-92, and miR-127, and a downregulation of four miRNAs: miR-92d-3p, miR-375-5p, miR-146-3p, and miR-694 were found in *S. iniae*-infected tilapia[41]. In addition, the activation of cellular and humoral immune responses, such as phagocytic cells, production of complements or cytokines, could play essential roles in bacterial destruction. Indeed, miR-29 has been shown to target IFN-γ, an important cytokine regulating innate immune responses to intracellular bacterial infection[36]. Similarly, miR-125 and miR-146 suppress excessive inflammation and maintain immunological homeostasis, which is critical for the outcome of pathogenic infections[28]. Additionally, an incubation of bovine monocytes with *Staphylococcus aureus* enterotoxin B led to lower the expressions of miR-125b, miR-155, and miR-223[9]. Furthermore, the downregulation of miR-24 and miR-181 in

human CD4 lymphocytes was associated with higher IFN-γ production[10]. Although extensive studies on the roles of these miRNAs in Nile tilapia have not been fully investigated, the results from other organisms, such as human, mice, or bovine could provide potential evidences on the regulatory functions of miRNAs on immune response during infection. Taken together, more information on the molecular targets and mechanisms of tilapia miR-29, miR-125, and miR-181 await further investigation.

In silico analysis showed that all three miRNAs complementarily bound to the target mRNAs of immune-related genes in Nile tilapia. Although the target gene prediction in this study was extrapolated from miRNA of the mouse (*Mus musculus*), the conserved sequence of miRNAs among vertebrates suggested that these target genes could be regulated by similar miRNAs in Nile tilapia. Interestingly, homologous genes, including *Ifi30*, *Cd276*, *Cdc42se1*, *Ddx3x*, and *Atg5*, were identified in the tilapia genome. Moreover, some of the mentioned genes have also been identified in teleosts. Such examples include *Cd276* in olive flounder (*Paralichthys olivaceus*)[19], *Lfng* in zebrafish[40], and *Ddx3x* in orange spotted grouper (*Epinephelus coioides*)[35]. Based on these findings, it is possible that these genes were expressed and could be targeted by miRNAs in tilapia.

In conclusion, this study highlighted on the conserved sequences of three miRNAs in Nile tilapia and other organisms. The qPCR protocols have been developed and validated to quantify the amount of miR-29, miR-125, and miR-181 expression in tilapia tissue. In fact, the R^2 values and melting curve analysis revealed the reproducibility and specificity of the assays. For further studies, qPCR protocols may pave the way to fully understanding the roles of these miRNAs in Nile tilapia. The downregulation of all three miRNAs were observed at 48–72 hr post Streptococcus-infection, suggesting that these miRNAs may participate in early immune regulation. Ultimately, understanding the biological functions of miRNAs could benefit the development of novel strategies for disease control in Nile tilapia.

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