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Decreasing salinity of seawater moderates immune response and increases survival rate of giant groupers post betanodavirus infection

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ABSTRACT

Giant groupers (*Epinephelus lanceolatus*), an important aquaculture fish in Asia, are attacked by nervous necrosis virus (NNV), belonging to betanodavirus. Environmental salinity can affect fish immunity and physiology. We examined whether decreasing salinity from 30 to 15 ppt during acclimation of groupers could affect survival with NNV infection and the associated factors. Although NNV infection decreased muscle moisture, up-regulated the gene expression of Na^+-K^+-$2Cl^-$ cotransporter isoform 2, and elevated plasma cortisol level in groupers, these factors were not related to the higher mortality of groupers reared at 30-ppt salinity (S30-groupers), compared to 15-ppt reared groupers (S15-groupers). Infected S30-groupers exhibited high leukocyte count and innate immune gene expression level. Moreover, NNV-infected dead S30-groupers showed high IL-1β gene expression level but low NNV load in the brain. The high or excess IL-1β gene expression levels in the brain of NNV-infected S30-groupers may be the factor in high mortality.

1. Introduction

Groupers (*Epinephelus* spp.) are valuable fish species in Asia aquaculture and widely distributed in tropical and subtropical areas. Viral nervous necrosis (VNN) disease, caused by nervous necrosis virus (NNV), belonging to the betanodavirus genus of *Nodaviridae* family, has caused mass mortality of groupers and resulted in severe economic loss [1]. NNV is an icosahedral and non-enveloped virion with a diameter of 20–34 nm and contains two single-stranded positive-sense RNAs, RNA1 and RNA2 [2,3]. RNA1 encodes RNA-dependent RNA polymerase (RdRp), and RNA2 encodes capsid protein. The clinical signs of NNV-infected fish include loss of equilibrium, spiral swimming behavior and darkening of body color. The histopathological characteristics are extensive vacuolation of nervous tissues, especially the brain and retina [1,4].

Groupers are euryhaline fish species and can live at the salinity of 10–33 parts per thousand (ppt). The optimal temperature for raising groupers is 22–28 °C. According to the experiences of our lab and grouper farmers, decreasing salinity seems to reduce the mortality of NNV-infected groupers. Therefore, this study aimed to confirm and clarify the influence of salinity on the mortality of groupers after NNV infection and to find out what factors are involved in the reduced mortality of NNV-infected groupers reared at low salinity of seawater. We previously used suppression subtractive hybridization (SSH) and found a high expression of Na^+-K^+-$2Cl^-$ cotransporter isoform 2 (NKCC2) gene in NNV-infected grouper larvae (unpublished data). The protein NKCC2 is present in the intestine of marine fish and mediates the absorption of Na^+, K^+, and Cl^- from imbibed seawater, accompanied by water absorption [5–8]. In this study, we cloned the giant grouper NKCC2 cDNA and determined the expression of NKCC2 gene expression and the muscle moisture of NNV-infected groupers to clarify whether NNV infection caused disordered osmoregulation and dehydration for up-regulated NKCC2 gene expression.

Besides affecting osmoregulation, salinity change can affect fish immunity and plasma cortisol concentration. High and low salinity in acclimating broad-nosed pipefish (*Syngnathus typhle*) increased the activity and proliferation of immune cells. Moreover, pipefish with *Vibrio* infection reared at low salinity showed lower immune cell proliferation and immune gene expression, compared with pipefish acclimated to ambient salinity [9]. Cortisol is the predominant corticosteroid in the teleost, and plays a critical role in

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growth, reproduction, and osmoregulation [10]. Plasma cortisol level is increased in fish under stress, such as handling and disturbance, rapid temperature changes, confrontations with predators, exposure to heavy metals, organic pollutants, and acid water [11,12]. Thus, the increase of plasma cortisol concentration is used as an indicator of stress in fish. Generally, in fish under acute stress, plasma cortisol levels elevate quickly within a few minutes and then are restored to normal levels some hours later. One study found that a sudden increase in salinity elevated plasma cortisol level in groupers [13]. In addition, if the stress is chronic, plasma cortisol may be maintained at a high level [12].

Because salinity can affect fish immunity and physiology, here we analyzed several immunity and physiological features of NNV-infected groupers acclimated to 15- and 30-ppt salinity. NNV-infected groupers usually die within 2 weeks during acute infection, so the innate immunity responding to acute infection may be related to the different mortalities of groupers reared at 15- and 30-ppt salinity (S15- and S30-groupers). Therefore, the expression of innate immune genes in NNV target organ, brain, and immune organs, head kidney and spleen, were analyzed, including pattern recognition receptors TLR-3 and MDA5 which can sense viral dsRNA, interferon-inducible antiviral protein Mx, pro-inflammatory cytokine IL-1β, and complement component 3 (C3). We determined the expression of NKCC2 and innate immune genes; blood cell counts; plasma cortisol concentration; and muscle moisture to investigate the possible factors associated with mortality of NNV-infected S15- and S30-groupers.

2. Materials and methods

2.1. Fish and virus

Two batches of giant groupers (*Epinephelus lanceolatus*) (mean body weight 25 g) were obtained from a hatchery farm in southern Taiwan and were used for NNV challenge tests I and II described in Section 2.3. The groupers were acclimated to 15- or 30-ppt salinity at 28 °C for 1 week before the experiment. We removed brains from a random sample of 5 groupers which were confirmed to be NNV-free by detecting NNV RNA2 through real-time PCR described in Section 2.4.

The NNV isolate, humpback grouper NNV (HGNNV), was propagated and titrated in GF-1 cells [14,15] and then stored at −80 °C.

2.2. Cloning of the full-length giant grouper NKCC2 cDNA

Total RNA was extracted from giant grouper intestine by the acid guanidinium thiocyanate-phenol-chloroform method [16]. For reverse transcription, total RNA (1 μg) was transcribed into cDNA by incubating at 42 °C for 1 h in 1× reaction buffer with a final volume of 30 μl containing 0.3 μM oligo dT_{20}, 0.4 mM dNTP, 11.7 mM DTT, 40 U ribonuclease inhibitor rRNasin (Promega) and 60 U MMLV reverse transcriptase (Promega). A 990-bp fragment of giant grouper NKCC2 gene was amplified by PCR with the primer set fNKCC-F and NKCC2-qR (Table 1). Primer fNKCC-F was designed from the gene of Japanese flounder fNKCC (AB618555), and primer NKCC2-qR was designed according to the fragment sequence of the giant grouper NKCC2 gene obtained from our previous SSH assay (unpublished data). PCR was carried out by adding an aliquot (5 μl) of the cDNA in the 1× PCR reaction buffer with a final volume of 25 μl containing 0.4 μM fNKCC-F and NKCC2-qR, 0.25 mM dNTP, and 0.5 U *Taq* DNA polymerase (NEB). PCR was performed in the Veriti 96 well thermal cycler (Applied Biosystems) with a denaturing step of 95 °C for 3 min, followed by 30 cycles of 95 °C for 30 s, 60 °C for 30 s, 68 °C for 90 s and a final extension of 68 °C for 10 min. The 990-bp fragment of giant grouper NKCC2 gene was cloned into pGEM-T easy vector (Promega) and sequenced.

Table 1
Primer sequences.

Primer name	Primer sequence
fNKCC-F	5′-CTC ATC AAT TTC TCC TGC TTC CAT GC-3′
NKCC2-sF	5′-ATG GAG ASV TTC AVK TCK ARC RRR GG-3′
5′-gsp1	5′- CGG GCC CAC AAA CAC TTC ACA GGT -3′
5′-gsp2	5′- GCT GGT CTG GCC CGT GCT GAA C-3′
3′-gsp1	5′- CCT CCG CCC CCG CAG GTT G-3′
NKCC2-qF	5′- GCA AGT GCT CAC GAC CAG AG-3′
NKCC2-qR	5′-AAA CAG CCA CCA CAC ATC AAT G-3′
NNV R3	5′-CGA GTC AAC ACG GGT GAA GA-3′
NNV-qF	5′-CAG TCC GAC CTC AGT ACA C-3′
NNV-qR	5′-AAC ACT CCA GCG ACA CAG-3′
Mx-F	5′-TGA GGA GAA GGT GCG TCC-3′
Mx-R	5′-GCG CCT CCA ACA CGG AGC TC-3′
MDA5-F	5′- CTG GGG TGG CTG AGA AGG AGT GT-3′
MDA5-R	5′- GCA GCT TCG GCA GGT AAA CTT CA-3′
TLR-3-F	5′-CTG GCT TAC TAC AAC CAC CCC-3′
TLR-3-R	5′-CAA ACT CCC TGC CCT CTT CA-3′
C3-F	5′-GGC GAC GGG CAA AAC CTA C-3′
C3-R	5′-GCT CCT CCA TGC CCA AAC AG-3′
IL-1β-F	5′-AGC GAC ATG GTG CGG TTT CTC-3′
IL-1β-R	5′-CTC TGT AGC GGC TGG TGG ACT C-3′
Actin-F	5′-GGC CGC GAC CTC ACA GAC TAC CTC-3′
Actin-R	5′-CCT CTG GGC AAC GGA ACC TCT CAT-3′

For cloning the 5′ terminal sequence of NKCC2 open reading frame, a degenerate forward primer, NKCC2-sF (Table 1), was designed according to the gene sequences from the start codon of fish NKCC2 (Table 2). Two gene-specific primers (gsp), 5′-gsp1 and 5′-gsp2 (Table 1), for amplifying 5′ terminal sequence of giant grouper NKCC2 gene were designed according to the 990-bp fragment of giant grouper NKCC2 gene. Total RNA of 6 μg was transcribed into cDNA by using the primer 5′-gsp1. An aliquot (5 μl) of the cDNA was amplified by PCR with NKCC2-sF and 5′-gsp1, and nested PCR was carried out using the primer set of NKCC2-sF and 5′-gsp2. PCR and Nested PCR were performed by initial denaturing step of 95 °C for 3 min, followed by 30 cycles of 95 °C for 30 s, 60 °C for 30 s, 68 °C for 4 min, with a final extension of 68 °C for 10 min. A 1946-bp PCR product was generated, cloned into pGEM-T easy vector (Promega) and sequenced.

For 3′ RACE, two primers, NKCC2-qF and 3′-gsp1 (Table 1), were designed from the 990-bp fragment of giant grouper NKCC2 gene. Total RNA of 6 μg was transcribed into cDNA by using the primer 3′-AP (Invitrogen). An aliquot (5 μl) of the cDNA was amplified by using the primer set NKCC2-qF and AUAP (Invitrogen). Touchdown PCR was performed by the protocol of denaturing at 95 °C for 3 min, followed by 5 cycles of 95 °C for 30 s, 68 °C for 30 s, 68 °C for 1 min, 5 cycles of 95 °C for 30 s, 65 °C for 30 s, 68 °C for 1 min, 25 cycles of 95 °C for 30 s, 60 °C for 30 s, 68 °C for 1 min, and with a final extension of 68 °C for 5 min. Nested touchdown PCR was carried out by the primer set 3′-gsp1 and AUAP (Invitrogen) with the PCR program of initial denaturing at 95 °C for 3 min, followed by 10 cycles of 95 °C for 30 s, 68 °C for 30 s, 68 °C for 1 min,, 25 cycles of 95 °C for 30 s, 65 °C for 30 s, 68 °C for 1 min, and with a final extension of 68 °C for 5 min. An 819-bp PCR product was cloned into pGEM-T easy vector (Promega) and sequenced. The sequences of the 5′ terminal PCR fragment and 3′ RACE overlapped the 990-bp NKCC2 partial sequence by 347 and 105 bp, respectively. The three parts of NKCC2 sequence were aligned to obtain the full-length cDNA of 3303 bp.

The giant grouper NKCC2 amino acid sequence was aligned with those of other species, and a phylogenetic tree (neighbor-joining) was created by using Clustal W in MEGA6, with bootstrapping values from 1000 replicates. The transmembrane domain was predicted by using the TMHMM server v2.0 (http://www.cbs.dtu.