Characterization of the Mx and hepcidin genes in *Epinephelus akaara* asymptomatic carriers of the nervous necrosis virus

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**A R T I C L E   I N F O**

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**A B S T R A C T**

Innate immunity plays a key role against the nervous necrosis virus (NNV) before adaptive immunity kicks off during fish development. We have studied the interferon-inducible Mx and the antimicrobial peptide hepcidin genes to probe the status of innate immunity in red-spotted groupers, *Epinephelus akaara* larvae and juveniles. For this study we examined the NNV infections in the farmed *E. akaara*, to generate the complete coding sequences of Mx and hepcidin, and to characterize the expression during development. Red-spotted grouper NNV (RGNNV) was mainly detected in the brain, gills and heart of the sick groupers, and the larvae from 0 to 23 day-post hatching (dph) of the farmed asymptomatic groupers were detected RGNNV-positive using real-time PCR (RT-PCR). The open reading frame (ORF) of *E. akaara* Mx gene is 1878 bp long encoding a putative protein of 626 amino acids, while the ORF of hepcidin is 258 bp in length encoding 86 amino acid residues. An RT-PCR was optimized to estimate the expression patterns of Mx and hepcidin in *E. akaara*. Mx was constitutively expressed in head kidney, liver, spleen, heart, gills, muscle, brain, thymus and intestine, and it was first detected on 2 dph and increased to a higher level after 15 dph. Hepcidin was mainly expressed in the liver and intestine, and it was detected in the fertilized-egg showing significantly increased expression after 29 dph. It could be hypothesized that the farmed groupers are RGNNV carriers, promoting vertical virus transmission from parents to the offspring, as the eggs were tested RGNNV positive. We can conclude that there should be a balance between the innate immunity and the RGNNV infection during early development, and RGNNV will outbreak when the innate immunity becomes weak and adaptive immunity is still immature.

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**1. Introduction**

In recent years, nervous necrosis virus (NNV) has caused a highly lethal disease in hatchery-reared larvae and juveniles of a wide range of marine fish around Asia, Europe, Australia, and America (Bovo et al., 1999; Comps et al., 1996; Curtis et al., 2001; Frerichs et al., 1991). Red-spotted grouper, *Epinephelus akaara* is listed as threatened on the International Union for Conservation of Nature (IUCN) Red List of Threatened Species based on the data available, showing a ~90% mortality during the larval stages in Chinese fish farms (Huang et al., 2005). Fish nodaviruses, from the genus *Betanodavirus*, are the causative agents of a highly lethal disease in hatchery-reared larval and juveniles of a wide range of marine fish. Since the complete sequence of the genomic RNA of nervous necrosis virus (RGNNV) was determined in 2012 (Liu et al., 2012), we have studied the possible reasons of the high mortality in *E. akaara* larvae focusing on the immune system level and taking into account the RGNNV infectivity.

Adaptive immunity and innate immunity play an important role against virus infection. In our previous study, RAG1 and IgM gene expression was observed at 15 days post-hatching (dph) and 23 dph respectively, increasing even to higher levels at 37 dph (Mao et al., 2012), which indicated that adaptive immunity of *E. akaara* develops its function around 30 dph. Innate immunity and maternal antibodies play important defensive roles before adaptive immunity starts to work. Type I interferon (IFN) response is one of the crucial defense lines against viral infection in vertebrates, and IFN inducible gene Mx has been isolated from various fish species. Antiviral role for Mx proteins has been well reported in fish (Abollo et al., 2005; Altmann et al., 2004; Chen et al., 2006; Fernandez-Trujillo et al., 2006; Jensen and Robertsen, 2000; Lee et al., 2000; Plant and Thune, 2004; Robertsen et al., 1997; Staeheli et al., 1989; Tafalla et al., 2004; Trobridge and Leong, 1995; Wu and Chi, 2007; Yap et al., 2003) and Mx has been used as a marker of type I IFN response in mammalians and fish.

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Mx proteins can localize either in the cytoplasm or in the nucleus, thus inhibiting the virus life cycle in different phases (Lee and Vidal, 2002). Recently, another innate immune-related factor hepcidin has been well studied in our lab (Peng et al., 2012). Hepcidin exerts not only antimicrobial but also antiviral functions (Cuesta et al., 2008). Hepcidin has been identified in many fish species, and the data reported demonstrates that hepcidin genes are common in mammals and fishes (Douglas et al., 2003; Hirono et al., 2005; Krause et al., 2000; Yang et al., 2007), indicating that this antimicrobial peptide is a very important component in the innate immune system. Besides, recent data shows that the maternal transfer of humoral innate and adaptive immune parameters of fish larvae seems to be responsible for the protection of embryos and larvae in the early stages of their development (Swain and Nayak, 2009). Here, Mx and hepcidin were characterized in the early developmental stages of red-spotted grouper as markers for the innate immune system.

2. Material and methods

2.1. Samples and RNA extraction

Eggs and larvae of *E. akaara* were collected from a hatchery in Zhangzhou, Fujian Province, China during the spawning season in 2011. While in the process of hatching, pools of 100 eggs and larvae at 0, 1, 4, 6, 9 and 12 dph were collected and head of larvae at 15, 18, 23 and 29 dph were sampled (n > 4). Larvae of 30 dph were maintained in the aquarium facilities in College of Ocean and Earth Sciences, Xiamen University, in a recirculation unit supplied with salt-water at 27 ± 3 °C, and fish were sampled on 37, 60 and 80 dph (n > 4). Samples of muscle, kidney, heart, thymus, brain, intestine, gonad, spleen, liver and gill (n > 4) were harvested from 4- and 18-month old groupers, respectively. RNA was immediately extracted from 50–100 mg of all the tissue samples mentioned above using the RNAprep pure tissue kit according to the manufacturer’s instructions (Tiangen Biotech) and reverse transcription (RT) was carried out using PrimeScript® RT reagent Kit (TaKaRa).

2.2. Confirmation and detection of RGNNV

Primers (qNNV) were designed based on the reported RGNNV sequences (GenBank accession no. AY744705.1) (Table 1). cDNA from brain was used as the template for PCR. PCR products were detected using electrophoresis with 1.2% agarose gel prepared with TAE buffer (Tris/acetate acid/EDTA), then purified from the gel using an AxyPrep DNA gel extraction kit (Axygen) and subcloned into pMD18-T vectors (TaKaRa) for sequencing (Invitrogen) to verify the cDNA sequence of the RGNNV.

![Fig. 1. Detection of RGNNV in *E. akaara*. A: Confirmation of RGNNV partial sequence. B: RGNNV distribution in the gonad (X), kidney (K), spleen (S), thymus (T), intestine (In), liver (L), heart (H), brain (B), gill (G) and muscle (M). C: Infection of RGNNV from fertilized eggs to 23 dph grouper larvae.](image-url)

Table 1

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequence (5′ → 3′)</th>
<th>Target</th>
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<tbody>
<tr>
<td>MxF1</td>
<td>GAATTCGGTTGCTACTGCTCT</td>
<td>Sequence of Mx cDNA</td>
</tr>
<tr>
<td>MxR1</td>
<td>AGTTTGTCCTCAATCCTCT</td>
<td>qPCR for Mx</td>
</tr>
<tr>
<td>MxF2</td>
<td>TTCATCCAATGCTCTCT</td>
<td>Hepcidin cDNA</td>
</tr>
<tr>
<td>MxR2</td>
<td>AACACGCAAACCACATCC</td>
<td>qPCR for hepcidin</td>
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<tr>
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<td>GCCATCTCAGTCTCTCT</td>
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<td>5′ adaptor</td>
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<td>25VN Universal primer of RACE PCR</td>
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<tr>
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<tr>
<td>qNNVR</td>
<td>GCATTTCCAGCAAACTTCTT</td>
<td>qPCR for NGNNV</td>
</tr>
</tbody>
</table>
All the samples mentioned in Section 2.1 were tested using RT-PCR with primers qNNV, and all of them tested NNV positive. Relative quantity of NNV was normalized against an endogenous reference β-actin (Mao et al., 2012) and presented as subtraction of target CT values from β-actin CT values (ΔCT value). Comparison of gene expression between tissues and calibrator was derived from subtraction of the calibrator ΔCT values from the target ΔCT values to give a ΔΔCT value, and relative gene expression was calculated to determine fold difference (2−ΔΔCT) (Wang et al., 2009).

2.3. Cloning and sequencing of Mx and hepcidin cDNA

Total RNA was extracted from the head kidney and the full-length sequence of Mx was deduced amino acid sequence shadowed below. The tripartite GTP-binding domain is boxed, the dynamin family signature is underlined, and the C-terminal leucine zipper motif is in black and italics.
Ends (3′-RACE) and 5′ RACE using commercial kits (Clontech) and following the procedures described by the manufacturer. Specific primers were designed based on the conserved regions of the known Mx sequences. The primer details are given in Table 1 and PCR conditions were as follows: 94 °C/4 min; 40 cycles of 94 °C/5 s, 55 °C/40 s, 72 °C/60 s; and 72 °C/10 min. cDNA PCR products were first detected using electrophoresis with 1.2% agarose gel prepared with TAE buffer (Tris/acetic acid/0.001% Na2EDTA) at 72 °C/10 min. cDNA PCR products were first detected using PCR amplification with primers as RGNNV Primer-fw and Primer-rv (Fig. 1A). All samples collected were identified as RGNNV-positive using RT-PCR (Fig. 1B), showing high levels of RGNNV in the head kidney, gill, and especially in the brain (Fig. 1C).

3.2. Isolation and characterization of E. akaara Mx

Fragments of E. akaara Mx gene were obtained by PCR and RACE PCR and composed to provide the full length cDNA sequence of 2201 bp (Fig. 2), which defined an open reading frame (ORF) of 1878 bp coding for a predicted protein of 626 amino acids with a tripartite GTP-binding domain, a dynamin family signature and a C-terminal leucine zipper motif (Fig. 2). The complete cDNA sequence of E. akaara Mx has been deposited in GenBank (GenBank accession no. JX683389).

Phylogenetic analysis revealed that E. akaara Mx was clustered with the Mx sequences from other teleosts (Fig. 3). In addition, there was a distinct separation between the teleost and mammalian Mx sequences. Alignment between the human and E. akaara Mx sequences highlights important conserved domains (Fig. 4A). For a better illustration, the sequence conservation of Mx protein was mapped onto the surface plot of the crystal structure of human MxA (PDB code 3SZR) using ConSurf (Fig. 4B) (Ashkenazy et al., 2010), using E. akaara as reference sequence. The conservation profile indicates that the higher conservation is located in the G domain and in the dimerization region within the stalk domain (Fig. 4B). Interestingly, the proline-induced hinge between the G-domain and the BSE domain is also conserved in E.akaara sequence (Fig. 4). All these features indicate function conservation.

3.3. Isolation and characterization of E. akaara hepcidin

The partial cDNA of E. akaara hepcidin sequence was 330 bp, including 35 bp of 5′ untranslated region (UTR), 258 bp of the ORF, and 37 bp of 3′ UTR (Fig. 5A) (GenBank accession no. JN596423). The gene encodes an 86 amino acid protein, which is composed of a signal peptide and a mature peptide. The signal peptide cleavage site was predicted between Ala 24 and Leu 25 (Fig. 5A).

A sequence alignment of E. akaara hepcidin precursor peptide and some other known and predicted hepcidin peptides was performed at the amino acid level (Fig. 5B). The alignment showed that the listed hepcidins were most characterized by eight conserved cysteine residues in the mature peptide region. There were eight ichthyic hepcidins sequences or ESTs with four cysteine residues in the mature peptide. The predicted signal peptide is highly conserved between E. akaara hepcidin and other fishes, however, the signal peptide cleavage sites are not conserved between mammalian and fish hepcidins (Fig. 5B).

3.4. E. akaara tissue expression of Mx and hepcidin genes

The mRNA transcript distribution of the Mx and hepcidin genes in the juvenile E. akaara was analyzed in various tissues using RT-PCR. Mx mRNA was detected in most tissues except the gonad (Fig. 6A), and the highest transcript levels were in the gill and liver. In comparison with the 4-month old groupers, Mx levels were significantly higher in the spleen but lower in the brain, head kidney and heart (P < 0.01) of the 18-month old groupers. Hepcidin mRNA transcripts were examined mainly in the liver of both 4- and 18-month old groupers. Weak signals were also detected in the thymus (2−ΔΔCT = 0.000586) and intestine (2−ΔΔCT = 0.001917) (Fig. 6B).

3.5. Expression pattern of Mx and hepcidin genes during development

The expression patterns of the Mx and hepcidin genes were investigated over different developmental stages, such as 0 (fertilized eggs), 1, 2, 4, 6, 9, 12, 15, 18, 23 and 29 dph of E. akaara using RT-PCR. β-actin was used as the endogenous control. Mx mRNA transcripts were first detected in the 2 dph group and increased significantly after 15 dph (Fig. 7A). Hepcidin mRNA transcripts were detected in the fertilized eggs and the levels increased after 23 dph (Fig. 7B).
**Fig. 4.** Mx protein features. A: Human and *E. akaroa* sequence alignment. B: Dimer representation of the Mx gene, in ribbon representation the most relevant protein domains are plotted. The sequence conservation of Mx protein is mapped onto the surface plot of the crystal structure of human MxA (PDB code 3SZR) using ConSurf. The degree of conservation follows a gradient from magenta, most conserved to cyan, least conserved. The highly conserved hinge inducing proline is indicated in green (corresponding to Pro340 in the MxA human sequence).
4. Discussion

4.1. RGNNV infection

RGNNV is the causal agent of viral encephalopathy and retinopathy (VER), a worldwide fish disease that induces high mortalities in marine species (Curtis et al., 2001; Munday and Nakai, 1997; Nakai et al., 1994; Xu et al., 2008). As reported, piscine nodaviruses have been classified into four major clades, i.e. jack nervous necrosis virus (SJNNV), red-spotted grouper nervous necrosis virus (RGNNV), tiger puffer nervous necrosis virus (TPNNV) and barfin flounder nervous necrosis virus (BFNNV) (Barke et al., 2002; Grotmol et al., 2000). Genome sequences of RGNNV have been determined, which consist of RNA1 and RNA2. RNA1 is 3103 nt in length and contains a 982-amino-acid open reading frame (ORF) encoding protein A, while RNA2 is 1433 nt long and contains a 338-amino-acid major ORF encoding the coat protein.

In this study, RGNNV was confirmed and detected in various tissues of *E. akaara* using RT-PCR. The level of RGNNV was significantly high in the brain, which is in accordance with VER symptoms. All larvae sampled from the farm were detected RGNNV positive, meeting the fact that a fraction of the offspring will behave as asymptomatic carrier, as this has been repeatedly reported for VNNV (Nishizawa et al., 1995). For the existence of an asymptomatic fraction, there should be a balance between the fish immunity and the NNV infection (Fig. 8). When fish immunity gets weak, the balance will be broken and the fish will die from NNV. The bias towards survival requires the compensation of the fish immune system. For this reason we probe the status of both adaptive (Mao et al., 2012), and innate (this study) immunity of *E. akaara*.

4.2. Characterization of Mx and hepcidin in *E. akaara*

Two cDNAs coding for Mx and hepcidin were cloned separately and characterized in red-spotted grouper. In the phylogenetic tree,
Mx shows high conservation in specific functional regions (Fig. 4), however, the evolutionary pressure on the Mx gene points towards the onset of two clusters, mammals vs. fishes (Fig. 3). Hepcidin seems to be a more environment dependent gene. Taking a look to the hepcidin phylogeny, we may observe three evolutive clusters – mammals, fish-hepcidin containing eight cysteines and fish-hepcidin containing four cysteines – depending on the most relevant functional unit, the cysteine-rich region (Fig. 5B). All hepcidin protein sequences show the basic residue patch characteristic of antimicrobial peptides. Arg/Lys rich areas are important to interact with membranes and produce the antimicrobial effect. From an evolution perspective, these innate immunity genes evolutionary profiles seem to be really driven by the specific environment (e.g. viral infections) as a selective pressure factor on the sequence divergence of these genes.

Fish Mx is an IFN-induced cytoplasmic protein with antiviral activity against a number of RNA viruses including orthomyxoviruses and rhabdoviruses (Horisberger et al., 1983; Meier et al., 1990). The available evidence suggests that triggering of IFN synthesis and induction of Mx may be important for the interaction of nodavirus proteins with the innate immune system (Magnadottir, 2006). Grouper cells overexpressing Mx are highly resistant to viral infection (Chen et al., 2008). Constitutive expression of Mx was found in gonad, kidney, spleen, intestine, liver, heart, brain, gill, muscle in E. akaara. A constitutive expression of Mx was also detected in a variety of tissues collected from different fish species. The results suggest that the antiviral activity of fish interferon system is ubiquitous in various tissues and cells. Besides, in the present study, it was found that the transcript levels of Mx in some tissues are different between the young and adult groupers. Mx expressing in 2 dph larvae means IFN system is independent of acquired immune system as it is still immature (Mao et al., 2012), which indicates that Mx plays an important protective role during early development stages.

E. akaara hepcidin possesses four cysteines in the mature region which are conserved in Antarctic fish and grouper hepcidins. To date, the biological function of hepcidins with four or eight cysteines is not clear yet. E. akaara hepcidin mRNA in the liver was at a high level in accord with the previous studies (Douglas et al., 2003; Yang et al., 2007). Hepcidin mRNA was observed in the fertilized-eggs, suggesting that this antimicrobial peptide helps to remove the invading pathogens, as well as regulating other immune-related components (Hancock and Scott, 2000).

5. Conclusion

In our previous study we showed that the onset of adaptive immunity of E. akaara was around 20 dph, represented by RAG1 and IgM expression (Mao et al., 2012). Important questions were how could the larvae be protected before the adaptive immunity onset and how could some of the juvenile survive while over 90% die? In this study, the results showed that the innate immune system...
represented by Mx and hepcidin works well before and after 20 dph, suggesting that innate immunity plays an important anti-RGNNV role before adaptive immunity develops. We can conclude that there exists a balance between RGNNV and fish immune system. The RGNNV carrier larvae survive while the immunity is strong enough, otherwise the balance would shift towards high mortality. It is still uncertain how long the immunity by maternal antibodies sustains, but it is sure that it turns weaker and weaker during development. Once the maternal antibody pool is exhausted and the fish adaptive immune system is still immature, there would be a “crisis period” where there are not enough anti-RGNNV antibodies (Fig. 8). However, the innate immunity of some groupers could work well against RGNNV infection to overcome this “crisis period” in order to develop adaptive immunity providing a survivor pool that gradually possesses effective specific antibodies to inhibit, and even scavenge, RGNNV.

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