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PATRÍCIA RAQUEL NOGUEIRA VIEIRA GIRÃO

UM ENFOQUE IMUNOLÓGICO SOBRE INFECÇÃO VIRAL EM *Litopenaeus vannamei* COLETADOS DE FAZENDAS DE CULTIVO NO NORDESTE DO BRASIL

FORTALEZA-CE

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Marinhas Tropicais do Instituto de Ciências do Mar (LABOMAR) da Universidade Federal do Ceará (UFC), como requisito parcial para a obtenção do grau de Mestre em Ciências Marinhas Tropicais.
Área de concentração: Oceanografia Biológica.
Linha de Pesquisa: Bioquímica e biotecnologia de recursos marinhos.
Orientador: Dr. Gandhi Rádis Baptista
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A Deus por me iluminar em todos os momentos,
a toda minha família pelo incentivo,
e a minha filha, Isabella, pela inspiração,

Dedico.

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RESUMO

O cultivo de camarão tornou-se uma importante indústria na aquicultura mundial. O Brasil está se tornando um dos principais produtores mundiais de camarão *Litopenaeus vannamei*. Entretanto, surtos de doenças virais estão afetando o setor aquícola em todo o mundo, causando perdas econômicas significativas. Entre os agentes virais que afetam os camarões marinhos, o vírus da necrose infecciosa hipodermal e hematopoiética (IHHNV) e o vírus da mionecrose infecciosa (IMNV) são os vírus epizoóticos mais prevalentes no Brasil. Dentro desse contexto, este trabalho teve como objetivo analisar amostras de camarão infectado por vírus coletadas de fazendas de cultivo no Nordeste do Brasil, bem como analisar os níveis de expressão de moléculas da imunidade dos animais durante a infecção. Após um período incomum de chuvas, em um programa de monitoramento de rotina para o diagnóstico de doenças, foram coletadas brânquias de camarões juvenis com sinais de doença viral. Para o diagnóstico da infecção foram utilizandas técnicas moleculares como PCR convencional, a transcrição reversa acoplada com PCR (RT-PCR) e PCR quantitativo (qPCR). Através da combinação das diferentes técnicas moleculares foi demonstrado que a maioria dos camarões doentes estavam co-infectados com ambos os vírus, IHHNV e IMNV. Este estudo foi o primeiro a demonstrar a ocorrência de uma co-infecção natural, causada por IHHNV e IMNV, em camarões peneídeos cultivados no nordeste do Brasil. Os valores recíprocos para carga viral sugeriram que pode está ocorrendo uma competição entre os dois vírus para infectar o hospedeiro. Para compreender como as moléculas-chave da imunidade inata respondem a esta dupla infecção, os níveis de HSP-70, crustinas, peneidinas-3a e lectina br-1 do tipo-C, foram avaliados por PCR quantitativo. Em testes de correlação linear, a HSP-70 apresentou a expressão regulada por IHHNV em brânquias de camarão duplamente infectado; no entanto, as transcrições dos demais genes analisados não apresentaram expressão regulada estatisticamente significativa. Esses resultados indicam que a HSP-70 pode ser um modulador diferencial de co-infecção viral no camarão, *L. vannamei*.

Palavras-chaves: *Litopenaeus vannamei*, IHHNV, IMNV, diagnóstico molecular, co-infecção viral, imunidade de camarões, qPCR

ABSTRACT

The shrimp farming has become an important aquaculture industry in the world. Brazil is becoming a leading global producer of shrimp *Litopenaeus vannamei*. However, outbreaks of viral diseases are affecting the aquaculture industry worldwide, causing significant economic losses. Among the viral agents that affect the marine shrimp Infectious hypodermal and hematopoietic necrosis virus (IHHNV) and infectious myonecrosis virus (IMNV) are prevalent epizootic viral agents in Brazil. Within this context, this study aimed to analyze samples of virus-infected shrimp collected from farms in northeastern Brazil, as well as analyzing the expression levels of molecules of immunity during infection of animals. After a period of unusual rains in a routine monitoring program for the diagnosis of diseases were collected gills of juvenile shrimp with signs of viral disease. For the diagnosis of infection were employed molecular techniques such as conventional PCR, reverse transcription coupled with PCR (RT-PCR) and quantitative PCR (qPCR). Through a combination of different molecular techniques has shown that most of shrimp patients were co-infected with both viruses, IHHNV and IMNV. This study was the first to demonstrate the occurrence of a natural co-infection, caused by IHHNV and IMNV in penaeid shrimp cultured in northeastern Brazil. The reciprocal values for viral load may suggest that competition is occurring between the two viruses to infect the host. To understand how the key molecules of innate immunity respond to this double infection, the level of HSP-70, crustin, peneidinas-3a and C-type lectin-br1 were assessed by quantitative PCR. In tests using linear correlation, HSP-70 expression is regulated by the presented IHHNV in doubly infected shrimp gills, however, transcripts of other genes analyzed showed no statistically significant regulated expression. These results suggest that HSP-70 may be a differential modulator co-infection viral in shrimp, *L. vannamei*.

Key-words: *Litopenaeus vannamei*, IHHNV, IMNV, molecular diagnostic, viral co-infection, shrimp immunity, qPCR

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LISTA DE ABREVIATURAS E SIGLAS

AMPs	Peptídeos antimicrobianos
cDNA	DNA complementar
CRDs	Domínios de reconhecimento de carboidrato
CTL	Lectinas do tipo C
CTLD	Proteínas contendo domínios semelhantes aos de lectinas do tipo C
DDRT-PCR	*Differential display reverse transcription-polymerase chain reaction*
ESTs	*Expressed sequence tags*
GAV	Vírus Associado à Brânquia
HSPs	Proteínas do choque-térmico
IHHN	Necrose infecciosa hipodermal e hematopoiética
IHHNV	Vírus da Necrose Infecciosa Hipodermal e Hematopoiética
IMN	Mionecrose infecciosa
IMNV	Vírus da Mionecrose Infecciosa
OIE	*Office International des Epizooties*
PAMPs	Moléculas padrões associadas aos patógenos
PRRs	Receptores de reconhecimento padrão
qRT-PCR	PCR quantitativo com transcrição reversa
SSH	*Suppression subtractive hybridization*
TSV	Vírus da Síndrome de Taura
WSSV	Vírus da Mancha Branca
YHV	Vírus da Cabeça Amarela

SUMÁRIO

1 INTRODUÇÃO

A aquicultura pode ser definida como o processo de produção de organismos aquáticos em cativeiro, dentre eles, peixes, crustáceos, moluscos, quelônios e anfíbios. Esse processo pode ser realizado no mar (maricultura) ou em águas continentais (aquicultura continental). A carcinicultura é o setor da aquicultura destinado à produção de crustáceos, seja de água doce, salgada ou salobra. Os camarões marinhos são os crustáceos mais cultivados em todo o mundo.

No Brasil, a carcinicultura comercial teve início no estado do Rio Grande do Norte, na década de 70. No mesmo período, o estado de Santa Catarina foi o primeiro a desenvolver pesquisas de reprodução, larvicultura e engorda do camarão cultivado e conseguiram produzir as primeiras pós-larvas em laboratório da América Latina. Na década de 90, com a importação da espécie exótica *Litopenaues vannamei*, a carcinicultura brasileira ganhou força, devido ao sucesso na adaptação dessa espécie no país. Atualmente camarões *L. vannamei* são a única espécie cultivada comercialmente no Brasil.

Fatores ambientais como, altas densidades, variações de salinidade e temperatura, tem sido frequentemente relatados como umas das razões para o surgimento de doenças no cultivo de camarões. Essas condições de estresse tornam os animais susceptíveis a uma grande variedade de doenças infecciosas. Os vírus estão entre os patógenos que tem causado significativas perdas econômicas na carcinicultura. No nordeste do Brasil, dois vírus constituem os principais causadores de doenças em *L. vannamei* cultivado: o Vírus da Mionecrose Infecciosa (IMNV) e o Vírus da Necrose Infecciosa Hipodermal e Hematopoiética (IHHNV).

Para diagnosticar camarões infectados, os métodos histológicos são utilizados para confirmar os sinais clínicos de viroses. No entanto, esta técnica é laboriosa, demorada e não são adequadas para detectar baixos níveis de carga viral. O desenvolvimento de técnicas mais eficazes e sensíveis, baseadas na tecnologia do DNA, como a reação em cadeia da polimerase (PCR) em tempo real e hibridização *in situ*, principalmente quando o objetivo é detectar a infecção ou obter reprodutores de camarão livre de patógenos.

No combate às infecções virais, bacterianas ou parasitárias, os camarões, dentre eles o *L. vannamei*, possuem um mecanismo de defesa baseado em componentes moleculares e celulares que, após a infecção, reconhecem padrões moleculares em membranas microbianas e contribuem para o desencadeamento de uma cascata de eventos que culminam com a morte dos agentes patogênicos.

Dentre as moléculas de reconhecimento, as lectinas do tipo C são amplamente estudadas em camarões e em muitos outros organismos, por representarem um papel importante na resposta imune. Outra classe de moléculas, são os peptídeos antimicrobianos (AMPs). As principais AMPs em camarões são as crustinas e peneidinas, moléculas-alvo de muitos trabalhos sobre atividade antimicrobiana, contra várias espécies de bactérias, pricipalmente *Vibrio*. Proteínas de grande significância durante uma infecção são as proteínas responsáveis pela manutenção da homeostase celular, como as proteínas do choque térmico (HSPs). Estas têm sido amplamente estudas em camarões, pois são proteínas que respondem a diversos estresses ambientais, bem como a invasões de patógenos.

Nos últimos anos, tem ocorrido um aumento de pesquisas sobre as interações patógeno-hospedeiro em camarões, entretanto, o conhecimento sobre os genes associados à resposta imune viral, bem como suas interações com o hospedeiro ainda é escasso. Assim, programas de controle dessas doenças, bem como manejo adequado, que inclui o uso de métodos diagnósticos de rotina, são de importância crucial para um bom desempenho da carcinicultura.

Visando contribuir para o melhor entendimento das respostas imunes do *L. vannamei* a infecções por IHHNV, IMNV ou ambos simultaneamente, faz-se necessário à realização de estudos sobre a correlação entre a replicação viral e as oscilações das moléculas-chave da imunidade dos animais durante uma infecção. Nesta dissertação foi realizado o diagnóstico e a quantificação dos vírus que infectavam *L. vannamei* cultivados no nordeste do Brasil, e simultaneamente as análises de expressão de crustina, peneidina, lectina do tipo C e HSP-70.

2 REVISÃO BIBLIOGRÁFICA

2.1 Camarão marinho cultivado: *Litopenaeus vannamei*

A produção de pescado mundial (captura e aquicultura) tem apresentado um crescimento considerável nas últimas décadas, enfim, tornando-se uma importante indústria para o suprimento de alimentos no mundo, com aproximadamente 154 milhões de toneladas somente no ano de 2011. A aquicultura representa uma maior parcela de contribuição para este crescimento, visto que a produção oriunda da captura permanece praticamente estável ao longo dos anos, devido ao declínio das populações silvestres (FAO, 2012).

O cultivo de organismos aquáticos em todo o mundo tem evoluído de quase insignificante até equiparar-se a produção pela pesca, alcançando em 2010 um máximo histórico desde a década de 90, com 79 milhões de toneladas (FAO, 2012). Dentre esses organismos, os crustáceos contribuíram com 9,7% da produção total no mundo. O crescimento desta atividade é mais proeminente em países tropicais e subtropicais (JOVENTINO & MAYORGA, 2008).

Os camarões peneídeos são os crustáceos responsáveis pelo maior volume de produção, aumentando a importância do cultivo de camarões e tornando-o um setor industrial relevante. Nas Américas, bem como em todo o mundo, essa atividade está baseada quase que inteiramente no cultivo do camarão branco do Pacífico, *Litopenaeus vannamei* (Figura 1), inclusive o Brasil, que tem se destacado na expansão desta atividade (FAO, 2012). Em 2004, empresários da indústria carcinicultora de quatorze países do ocidente produziram mais de 200 mil toneladas de camarão, proporcionando grande receita e empregando muitas pessoas (LIGHTNER, 2011).

Figura 1 – *Litopenaeus vannamei*.
Fonte: www.ictioterm.es

No Brasil, a produção de pescado decorrente da aquicultura representa em torno de 27%. Dentro da maricultura brasileira, a produção de camarões contribui com 82,9% da

produção total. Os estados de Rio Grande do Norte e Ceará são os maiores produtores de *L. vannamei* do Brasil (BRASIL, 2007; ABCC, 2011).

Os crustáceos são um grupo de invertebrados constituídos por mais de 42.000 espécies com a maioria aquática (HICKMAN *et al.*, 2004). Este grupo inclui inúmeras espécies apreciadas para consumo humano e de alto valor comercial, tais como camarões, lagostas, lagostins, caranguejos e siris. Estes organismos vêm sofrendo uma forte pressão pela pesca e consequentemente, ameaçando a manutenção dos estoques naturais (FAO, 2012). O cultivo de crustáceos, principalmente de camarões, desponta como uma importante alternativa para a produção rápida e em larga escala de alimento humano com alta qualidade, auxiliando ainda a proteger as populações naturais de um esgotamento das populações naturais (BRASIL, 2007).

A espécie *L. vannamei* pertence ao reino Animalia e sua classificação taxonômica segue o esquema abaixo:

Filo Arthropoda
Subfilo Crustacea - Brünnich, 1772
Classe Malacostraca - Latreille, 1802
Subclasse Eumalacostraca - Grobben, 1892
Superordem Eucarida - Calman, 1904
Ordem Decapoda - Latreille, 1802
Subordem Dendrobranchiata - Bate, 1888
Superfamília Penaeoidea - Rafinesque, 1815
Família Penaeidae - Rafinesque, 1815
Gênero *Litopenaeus* - Pérez Farfante, 1969
Espécie *Litopenaeus vannamei* - Boone, 1931

Essa espécie se distribui desde Sonora (norte do México) até o Tumbes (norte do Peru), normalmente em águas tropicais (BARBIERI & OSTRESKY, 2002). Características como rusticidade, rápido crescimento, fácil adaptação a rações comerciais e boa tolerância a variações ambientais, fizeram do *L. vannamei* o camarão mais cultivado em todo o mundo (BRASIL, 2007).

2.2 Enfermidades virais em camarões

O cultivo de camarões marinhos têm sofrido problemas recorrentes de surtos de doenças, causando consequentemente grandes perdas econômicas, por exemplo, em Moçambique no ano de 2011, praticamente anulou esta atividade no local (FAO, 2012).

Diversas situações propiciam o desenvolvimento de doenças nos viveiros de cultivo, como altas densidades populacionais e variações bruscas de fatores físico-químicos (salinidade, temperatura entre outros) (BACHÈRE, 2000). O controle de infecções, principalmente virais, tem sido tratado como um ponto crítico para o bom desempenho produtivo da carcinicultura (BARRACCO, 2004). Assim, programas de controles dessas doenças, bem como manejo adequado, que inclui o uso de métodos diagnósticos de rotina, são de importância crucial para a carcinicultura.

A etiologia de doenças infecciosas de importância econômica para camarões cultivados podem ser de origem variada como: bactérias, fungos, protozoários e vírus. Muitas das doenças causadas por bactérias, fungos e protozoários são controladas utilizando boas práticas de manejo e quimioterápicos. Por outro lado, as doenças virais têm sido mais difíceis de gerenciar, pois, uma vez instaladas não existem métodos eficazes de tratamento (BACHÈRE, 2000).

Os vírus que mais prejudicam o cultivo de camarões peneídeos em todo o mundo são: vírus da mancha branca (WSSV), vírus da necrose infecciosa hipodermal e hematopoiética (IHHNV), vírus da síndrome de taura (TSV), vírus da cabeça amarela (YHV) e vírus da mionecrose infecciosa (IMNV) (LIGHTNER, 2012).

No nordeste do Brasil, dois vírus constituem os principais causadores de doenças em *L. vannamei* cultivados, o IMNV e o IHHNV (POULOS & LIGHTNER, 2006).

2.2.1 IHHNV

A necrose infecciosa hipodermal e hematopoiética (IHHN) é causada por um parvovírus estável da família Parvoviridae e um provável membro do gênero Brevidensovirus, o IHHNV (Figura 2). Este é um vírus icosaédrico, não-envelopado, com diâmetro de 22 ηm e com material genético composto por DNA genômico de fita simples com 4,1 kb (BONAMI *et al.*,1990). O genoma do IHHNV codifica três fases de leitura aberta (do inglês ORFs - *open reading frames*), ORF 1 que corresponde a uma proteína não-estrutural, ORF 2 e e ORF 3 uma proteína superficial de 37 kDa (BONAMI *et al.*, 1990; MARI *et al.*, 1993).

O IHHNV é vírus ubíquo que acomete várias espécies de camarões peneídeos da Ásia, América Central e América do Sul. Entretanto, foi isolado pela primeira vez em juvenis de camarão azul, *Penaeus* (*Litopenaeus*) *stylirostris*, no Havaí, em 1981 (LIGHTNER *et al.*, 1983). No caso de *L. vannamei*, apesar de taxas relativamente baixas de debilidade e mortalidade causadas pelo IHHNV, a manifestação típica da doença inclui retardo de crescimento, que é caracterizado por altos níveis de consumo de energia, e a síndrome da deformidade e do nanismo, resultando em deformidades cuticulares e rostro dobrado (KALAGAYAN *et al.*, 1991). Esses sintomas podem ser responsáveis por decréscimos na produção de camarão comercial no mercado, além do grande desperdício de nutrientes (ração) para o crescimento do crustáceo.

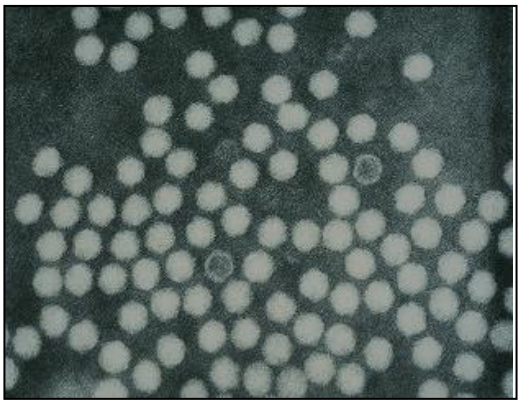

Figura 2 – Eletromicrografia do IHHNV.
Fonte: (LIGHTNER *et al.*, 2012).

Podem ocorrer três variantes genéticas do IHHNV, sendo essas: IHHNV – I, presente nas Américas e Filipinas; IHHNV – II, presente na Ásia; IHHNV – III, encontrada na África e Austrália. Os dois primeiros genótipos são infecciosos para *L. vannamei* e *Penaneus monodon*, enquanto o terceiro não é infeccioso para essas espécies, pois sequências relacionadas ao IHHNV – III foram detectadas integradas ao genoma de algumas populações geneticamente diferentes de *P. monodon* na região Indo-Pacífica (LIGHTNER, 2011).

As formas de transmissão da doença podem ser verticais e horizontais. A forma vertical dá-se pela herança genética de fêmeas ou machos infectados. A horizontal ocorre por água contaminada, vetores (aves aquáticas) e/ou canibalismo. (MOTTE *et al.*, 2003). Algumas aves aquáticas, como as gaivotas, transmitem o IHHNV através das fezes (VANPATTEN *et al.*, 2004).

2.2.2 IMNV

Em 2002, o IMNV foi identificado como o agente causador de uma doença no músculo do camarão cultivado no nordeste do Brasil. O vírus se disseminou rapidamente para outras regiões do Brasil e, em 2003, foi responsável por uma perda econômica avaliada em alguns milhões de dólares (OIE, 2009). Fora do Brasil, foram confirmados surtos de IMNV em cultivo de *L. vannamei* na Indonésia (SENAPIN *et al.*, 2007).

O IMNV é um provável membro da família Totiviridae. Este vírus também apresenta simetria icosaédrica, não-envelopado e possui 40 ηm de diâmetro (Figura 3). O seu genoma é composto de RNA dupla fita com 7560 pb. O sequenciamento do genoma viral indicou a presença de dois ORFs. O ORF 1, que codifica as proteínas do capsídeo e a RNA-*binding*, e ORF 2 que codifica a RNA polimerase RNA-dependente (RdRp) (POULOS *et al.*, 2006). Além disso, foi demonstrado por reconstrução de imagem tridimensional do IMNV uma proteína do capsídeo de 120 kDa semelhante a estrutura do totivirus (TANG *et al.*, 2008).

Os sintomas característicos da doença causada por IMNV incluem: a perda de volume do hepatopâncreas; perda de transparência e coloração, principalmente no abdômen distal e em torno da cauda; necrose dos músculos estriados do abdômen, apêndices e cefalotórax; regiões esbranquiçadas na musculatura e necrose progressiva da nadadeira caudal. Podendo ocorrer, em alguns estágios do crescimento do camarão, morbidade e mortalidade, porém camarões juvenis são mais susceptíveis a mortalidade. O estresse causado por condições ambientais desfavoráveis parece ser um fator de desencadeamento da infecção tanto aguda como crônica (LIGHTNER *et al.*, 2004).

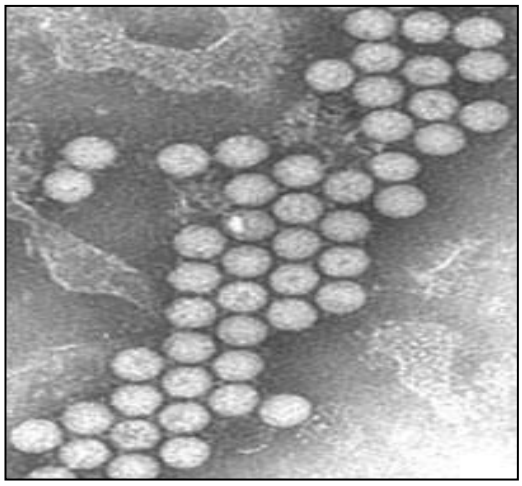

Figura 3 – Eletromicrografia do IMNV.
Fonte: (POULOS *et al.*, 2006).

Em estudos anteriores, foi detectado, no Laboratório de Bioquímica e Biotecnologia do Instituto de Ciências do Mar (UFC), a ocorrência de co-infecção natural com IHHNV e IMNV em *L. vannamei* de fazendas de cultivo no nordeste do Brasil (TEIXEIRA *et al.*, 2010). Neste estudo, as análises, baseadas em PCR convencional, provaram que 98% dos camarões eram portadores de IHHNV assintomáticos, pois os sinais grosseiros da infecção não foram manifestados quando realizada a amostragem.

A prevalência de co-infecção de *L. vannamei* com IHHNV e outros vírus, como o TSV ou WSSV (YU *et al.*, 2011), e ainda em associação com esses dois vírus (TSV e WSSV), ou seja, uma tripla-infecção dos vírus, foi encontrada entre as amostras de camarão em diferentes fazendas de camarão localizadas na China (TAN *et al.*, 2009).

2.3 Imunidade de crustáceos

Os crustáceos não possuem sistema imune adaptativo (adquirido), ou seja, o seu mecanismo de defesa consiste de várias respostas imunes inatas. Este sistema é baseado em componentes moleculares e celulares (hemócitos) que, após a entrada do patógeno, reconhecem padrões moleculares nas membranas de agentes infecciosos e contribuem para o desencadeamento de uma cascata de eventos químico-enzimáticos que culminam com a morte dos agentes patogênicos (BACHÈRE, 2000; IWANAGA & LEE 2005).

Em crustáceos, existem três tipos de hemócitos: hemócitos hialinos, hemócitos semi-granulares ou com grânulos pequenos e hemócitos granulares ou com grânulos grandes (VAN DE BRAAK *et al.*, 2002). A ativação dos hemócitos resulta geralmente em sua migração da hemolinfa para o tecido e degranulação, havendo a liberação de uma grande variedade de efetores imunológicos para o músculo. Estas células estão envolvidas principalmente com fagocitose, produção de lectinas, encapsulação, coagulação e ativação do sistema pró-fenoloxidase (BARRACO, 2004).

Algumas moléculas fazem parte da resposta imune inata, como por exemplo, as lectinas do tipo C (CTL), são proteínas dependentes de cálcio com um ou mais domínios conservados de reconhecimento de carboidrato (CRDs). As CRDs agem como receptores de reconhecimento padrão (PRRs) ligando-se as moléculas padrões associadas aos patógenos (PAMPs) ativando a defesa inata do hospedeiro (FUJITA *et al.*, 2004). Lectinas do tipo C e proteínas contendo domínios semelhantes aos de lectinas do tipo C (CTLD) tem sido identificadas em várias espécies de crustáceos, incluindo caranguejos (KONG *et al.*, 2008) e camarões (LUO *et al.*, 2006; LIU *et al.*, 2007; COSTA *et al.*, 2011), bem como em outros

invertebrados como: artrópodes (MATSUBARA *et al.*, 2007), cnidários (WOOD-CHARLSON & WEIS, 2009) e moluscos (YAMAMURA *et al.*, 2008; ZHANG *et al.*, 2009).

Outros polipeptídeos que agem conjuntamente na defesa inata em camarões peneídeos, incluem membros da família de peptídeos antimicrobianos (AMPs) e proteínas do choque térmico (HSPs).

Os AMPs são moléculas catiônicas, ricas em resíduos de prolina e cisteína, possuindo, geralmente, baixo peso molecular (abaixo de 10 kDa). O efeito microbicida ocorre pela formação de poros na bicamada lipídica das membranas bacterianas, alterando seu equilíbrio iônico, ou ainda pela interferência na biossíntese da membrana do microrganismo, provocando sua lise (BACHÈRE, 2000). Por exemplo, crustinas e peneidinas (PEN) compreendem duas classes de AMPs que são expressos em hemócitos de crustáceos e camarões peneídeos (MUÑOZ *et al.*, 2002).

As crustinas, chamadas coletivamente de carcininas, foram identificadas inicialmente no caraguejo *Carcinus maemas*, (SCHNAPP *et al.*, 1996). Em *L. vannamei* e *L. setiferus*, mais de uma isoforma tem sido encontrada (GROSS *et al.*, 2001). As crustinas são classificadas em três tipos (I, II e III) de acordo com suas funções e a disposição dos seus domínios proteicos (SMITH *et al.*, 2008).

A família de peneidinas também inclui membros com funções e sequências de aminoácidos variáveis. Três famílias que mostram atividade biológica contra fungos e bactérias têm sido caracterizadas de hemolinfa de *L. vannamei* (DESTOUMIEUX *et al.*, 1997). Em *L. vannamei*, foi demonstrado o espectro de atividade das peneidinas, identificados seus genes codificadores e caracterizada a regulação de sua expressão (MUÑOZ *et al.*, 2002, 2003, 2004).

Outras classes de polipetídeos que são recrutados em resposta a um estímulo externo e estão presentes no sistema imune de crustáceos são as HSPs. As HSPs tem a função de manter a conformação natural das proteínas celulares, agindo como chaperonas intrínsecas, e incluem proteínas com peso molecular variando de 16 a 100 kDa, correspondendo ao nome da família a que pertence (por exemplo, HSP21, HSP60, HSP70 e HSP90), sendo expressas por todos os organismos celulares em resposta não somente ao estresse térmico, como também ao ataque de patógenos, estresse oxidativo e osmótico, exposição a agentes tóxicos (ZHOU *et al.*, 2008; ROBERTS *et al.*, 2010; ZHOU *et al.*, 2010a). Em *Penaues monodon*, as HSPs foram previamente caracterizadas pela identificação de sequências de cDNA de HSP21, HPS70 e HSP90 (JIAO *et al.*, 2004; WU *et al.*, 2008; ZHOU *et al.*, 2010b; HUANG *et al.*, 2011).

2.4 Estudo da expressão de genes relacionados à imunidade em camarões

Para melhor entender a patogênese de qualquer doença, o conhecimento entre as interações patógeno e hospedeiro é um ponto crítico. A relação vírus-hospedeiro pode resultar em resposta de defesa contra o invasor, bem como ocasionar mudanças metabólicas e moleculares na maquinaria celular, alterando o nível de expressão de determinados genes que favorecem a replicação viral (WANG & ZHANG 2008).

O desenvolvimento de ensaios quantitativos para a avaliação e o monitoramento do estado imune de camarões cultivados é crucial para a prevenção de doenças (BACHÈRE, 2000). Neste sentido, para a obtenção de dados mais robustos de respostas imunes de camarões infectados, técnicas como PCR quantitativo em tempo real (qRT-PCR) e microarranjos de cDNA são mais indicadas (YUE *et al.*, 2006; ROSA *et al.*, 2007; QIAN *et al.*, 2012). Essas técnicas têm sido amplamente utilizadas para avaliar a oscilação de genes da imunidade em crustáceos, bem como para biomarcadores de poluição e estresse ambiental (ROSA *et al.*, 2007; YAN *et al.*, 2010).

Na PCR em tempo real, o acúmulo de produtos da PCR é detectado e monitorado na fase inicial da reação, diretamente pela leitura do aumento da fluorescência do composto fluorescente utilizado, enquanto na PCR tradicional os produtos são analisados quando a PCR já atingiu sua fase platô. O principal fundamento na utilização deste método é o chamado *threshold cycle* (CT) (Figura 4). O CT é definido como o número de ciclos calculado no qual o produto de PCR atinge um limiar de detecção, ou seja, o sinal fluorescente do corante sinalizador atravessa uma linha arbitrária denominada *threshold*. O valor numérico do CT é inversamente proporcional à quantidade inicial do transcrito de interesse na reação, ou seja, quanto menor o valor de CT, maior a quantidade inicial do transcrito na amostra (WONG & MEDRANO, 2005).

A quantificação gênica pode ser feita de duas formas: quantificação absoluta e quantificação relativa. A quantificação absoluta determina o número exato de cópias do gene de interesse, através da comparação com uma curva padrão. Esta metodologia é muito utilizada na quantificação viral (BUSTIN *et al.*, 2009; PFAFFL, 2001).

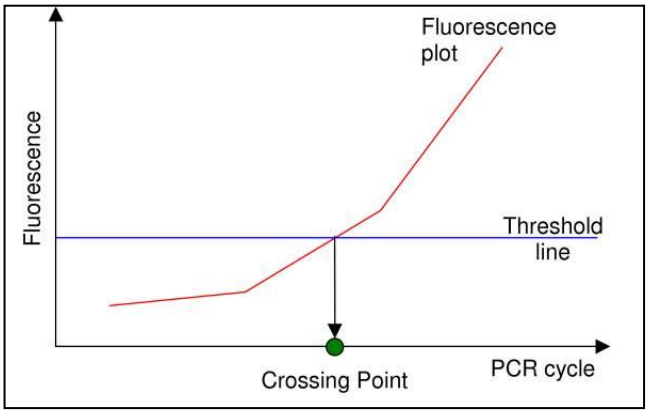

Figura 4 – Esquema indicando o *Threshold Cycle* (CT).
Fonte: LARIONOV *et al.*, 2005.

Diferente da PCR convencional, a PCR em tempo real utiliza o composto fluorescente diretamente na reação antes de ocorre a ciclagem no termociclador. Vários métodos de fluorescência têm sido desenvolvidos. Alguns se baseiam na utilização de sondas complementares ao gene de interesse, como por exemplo, as sondas *Taqman* (YUE *et al.*, 2006). A detecção baseada em sondas assegura que o único produto detectado é o produto de interesse, produtos não-específicos e dímeros de iniciadores não podem ser detectados. Vários corantes estão disponíveis, permitindo a distinção entre diferentes cores, sendo possível a detecção simultânea de múltiplos produtos de PCR (PCR multiplex), chegando a detectar a amplificação de até cinco genes de interesse diferentes e simultaneamente em uma mesma reação.

Outro método de detecção é a utilização do SYBR Green I (Figura 5), um corante fluorescente que se liga na volta menor do DNA (SIMPSON *et al.*, 2000). Em solução, a sua fluorescência é baixa, mas aumenta com a ligação ao DNA dupla fita. Das químicas de detecção disponíveis, esta é a mais barata, não necessitando de sondas específicas e podendo ser usada com qualquer iniciador. Uma desvantagem do SYBR Green I é a sua inespecificidade, visto que pode se ligar não somente ao produto de interesse, mas também a produtos inespecíficos e dímeros de iniciadores. Porém, este problema pode ser evitado desenhando-se iniciadores mais específicos e através da análise da curva de dissociação dos produtos (*melting curve*). No final da amplificação, a temperatura do instrumento é regulada para 5 °C abaixo da temperatura de anelamento, permitindo que todos os produtos dupla-fita se anelem ao SYBR Green I, obtendo um sinal máximo de fluorescência. O aparelho é então programado para aumentar a temperatura lentamente (0,1-1 °C/s) enquanto o sinal de fluorescência é constantemente monitorado. Se somente um produto foi gerado durante a reação de PCR, a fluorescência medida irá cair dramaticamente assim que todos *amplicons*

idênticos desnaturarem, ou seja, quando a temperatura de *melting* dos produtos é atingida, gerando um pico para *amplicons* idênticos. Se dímeros de iniciadores são formados, estes geralmente produzem o pico de *melting* em uma temperatura mais baixa, já que são de tamanho menor que os produtos verdadeiros (ZIPPER *et al.*, 2004).

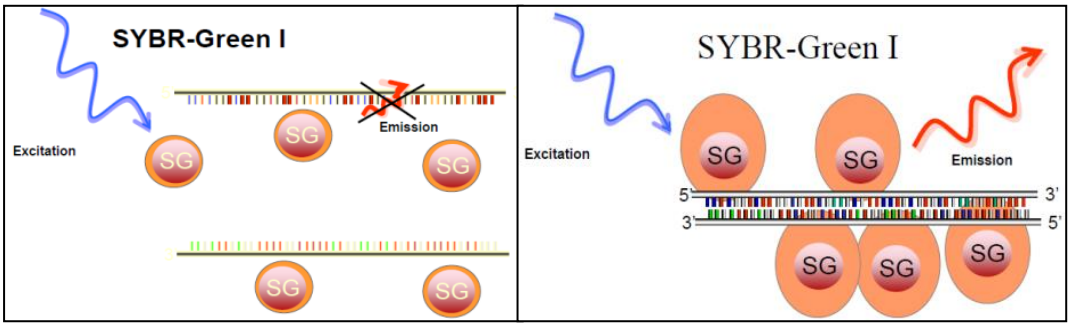

Figura 5 – Uso do SYBR Green I para detectar produtos em qRT-PCR.
Fonte: Cobertt Research.

Várias outras técnicas são também utilizadas para analisar genes diferencialmente expressos tais como: *Differential display reverse transcription-polymerase chain reaction* (DDRT-PCR) (KAMIMURA *et al.*, 2008); *Suppression subtractive hybridization* (SSH) (PAN *et al.*, 2005) e *Expressed sequence tags* (ESTs) (CLAVERO-SALAS *et al.*, 2007; LI *et al.*, 2012).

A identificação e quantificação da expressão gênica de diferentes moléculas imunológicas em crustáceos têm auxiliado significativamente na compreensão das respostas imunes desencadeadas nos processos infecciosos, assim como dos mecanismos relacionados à interação patógeno-hospedeiro, ainda pouco conhecido (BACHÈRE, 2000). Esses estudos também auxiliam na descoberta de ferramentas promissoras para biomonitoramento de condições ambientais e de saúde de camarões cultivados, pois a partir de uma compreensão de como o animal responde ao estresse ambiental, isto implicará em melhorias nas condições da aquicultura para aumentar a imunidade do camarão, protegendo-os de doenças (QIAN *et al.*, 2012).

Para avaliar moléculas relacionadas à imunidade, em epipodito do camarão *Penaeus monodon*, moléculas semelhantes à crustina foram estudadas e apresentaram sua expressão aumentada após o tratamento térmico e estresse salino hiperosmótico. Estes aumentos na expressão de moléculas semelhantes à crustina funcionam com mediadores do estresse, além da sua atividade antibacteriana *in vitro* contra bactérias Gram-positivas (VATANAVICHARN *et al.*, 2009). Quanto as peneidinas, o perfil de expressão de PEN-2, -3 e -4 em hemócitos de

L. vannamei foram estudadas por PCR quantitativo (qPCR) e apresentaram flutuações, onde a PEN-3 foi expressa 1000 vezes mais do que PEN-2 e PEN-4 (O'LEARY & GROSS, 2006).

Com a intenção de contribuir para o melhor entendimento da resposta ao estresse e consequentemente a imunidade, os níveis de três famílias de HSPs foram estudas em vários tecidos de *P. monodon* e a família com níveis basais mais altos foi a HSP70, seguida pela HSP90 e HSP21. Em resposta ao choque-térmico e a exposição ao *Vibrio harveyi*, todas as três HSPs tiveram sua expressão induzida (RUNGRASSAMEE *et al.*, 2010). O mesmo foi observado para infecções virais, onde o aumento da expressão de HSP70 foi correlacionado a redução na taxa de replicação de um vírus que infecta o camarão (vírus associado à brânquia, GAV) (VEGA *et al.*, 2006).

Em *L. vannamei*, o perfil de expressão de HSP60 e HSP70 em resposta à infecção bacteriana e ao choque térmico tem sido estudado e mostrou que ambas as proteínas tem a expressão aumentada em tecidos de brânquia, hepatopâncreas e hemócitos após a exposição dos camarões a bactérias como *Staphylococcus aureus* e *Vibrio alginolyticus* (ZHOU *et al.*, 2010b).

A HSP70 tem sua expressão bastante sensível as mais diversas variações de condições ambientais, em *L. vannamei*, com destaque para o estresse térmico, bem como a exposição a metais pesados, em especial ao ferro e zinco (QIAN *et al.*, 2012). Considerando que distúrbios bruscos, nas características físico-químicas, do ambiente constituem as principais razões que os camarões se tornam mais suscetíveis a doenças infecciosas em condições de cultivo, as HSPs despontam como candidatos a biomarcadores de estresse e, consequentemente, de resposta imune às doenças, podendo ser utilizados como marcadores do estado de saúde dos camarões cultivados (WU *et al.*, 2008).

3 JUSTICATIVA

As perdas econômicas oriundas de infecções virais em fazendas de cultivo de camarão marinho têm preocupado os empresários da carcinicultura mundial. No Nordeste do Brasil, já ocorreram várias perdas significativas na produção de *L. vannamei* devido a infecções por IHHNV e IMNV. Ao contrário de doenças bacterianas, não há fármacos para o tratamento de infecções causadas por vírus em camarão. Por esse motivo, exames de diagnósticos rotineiros têm sido realizados em muitas fazendas de cultivo, para monitorar e prevenir o aparecimento dessas doenças. Entretanto, os exames comumente realizados não identificam indicadores bioquímicos internos do estado de saúde dos camarões, presentes desde estágios iniciais da infecção.

Para monitorar o estado de saúde dos camarões cultivados, e, consequentemente, prevenir o aparecimento de surtos de doenças em fazendas de cultivo de camarões, fazem-se necessários estudos das interações vírus-hospedeiro. Nesse contexto, o monitoramento de infecções virais, bem como a resposta de moléculas-chave do sistema imunológico do camarão, é fundamental para o bom desempenho da carcinicultura em nossa região.

4 HIPÓTESE

As moléculas responsáveis pela resposta imunológica, como crustinas, peneidinas, lectinas do tipo C e HSPs oscilam de acordo com a quantidade e o tipo de vírus causador da infecção em *L. vannamei* cultivados.

5 OBJETIVOS

5.1 Objetivo geral

Realizar um estudo sobre a resposta imune de *L. vannamei* frente a infecções causadas pelos vírus, IHHNV e IMNV, em fazendas de cultivo no nordeste do Brasil.

5.2 Objetivos específicos

1 Monitorar a ocorrência de doenças virais em fazenda de cultivo de *L. vannamei* no nordeste do Brasil;

2 Diagnosticar o agente causador da doença observada em viveiros de cultivo de *L. vannamei* por técnicas moleculares, como PCR e RT-PCR;

3 Quantificar a carga de vírus em brânquias coletadas de camarões com sinais de doença viral;

4 Quantificar o nível de expressão de crustina, peneidina-3, lectina do tipo C (CTL-br1) e HSP-70;

5 Correlacionar os níveis de expressão das moléculas imunes estudadas com a quantidade de cada vírus separadamente.

6 CAPÍTULO I

Natural co-infection with infectious hypodermal and hematopoietic necrosis virus (IHHNV) and infectious myonecrosis virus (IMNV) in *Litopenaeus vannamei* in Brazil

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Aquaculture

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Abstract

Cultivation of whiteleg shrimp (*Litopenaeus vannamei*) constitutes a growing aquaculture industry in northeast Brazil. Similar to other animals that are intensively farmed, shrimp experience disease outbreaks, which are a constant threat and cause eventually significant economical losses. Infectious hypodermal and hematopoietic necrosis virus (IHHNV) and infectious myonecrosis virus (IMNV) are prevalent epizootic viral agents in Brazil. In a routine monitoring program for the diagnosis of IHHNV and IMNV, using molecular techniques like conventional PCR, reverse transcription coupled with PCR (RT-PCR) and absolute quantitative PCR (qPCR), we found that most positive samples of shrimp were simultaneously co-infected with both viruses. This survey is the first to show the occurrence of a natural co-infection that is caused by IHHNV and IMNV in penaeid shrimp attacked by viral disease that were cultivated in Northeast Brazil.

Keywords: brazilian aquaculture, shrimp virus, viral co-infection, molecular diagnostic, IHHNV, IMNV, qPCR.

1. Introduction

According to the Food and Agricultural Organization (FAO, 2010), the whiteleg shrimp (*Litopenaeus vannamei*) is among the six most cultured aquatic species in the world. In 2007, whiteleg shrimp production totaled more than 2.3 billion tons, and revenue accounted for almost US$10 billion. Brazil is one of the main global producers of this shrimp species. Approximately 98% of Brazil's shrimp aquaculture production is concentrated in the northeastern region of the country. The climate, characterized by low temperature fluctuations (between 22 °C and 30 °C) and low rainfall throughout the year, is one of the main factors that favor shrimp and fish aquaculture in the Brazilian Northeast.

Diseases constitute a permanent threat to the shrimp aquaculture industry and significant economic losses occur episodically (Lightner et al., 1997; Lightner and Redman, 1998; Brock and Bullis, 2001; Lightner 2003). Two viruses are the primary causative agents of epizootic diseases of *L. vannamei* farmed in the northeast of Brazil, namely, infectious hypodermal and hematopoietic necrosis virus (IHHNV) and infectious myonecrosis virus (IMNV).

IHHNV was first detected in *L. stylirostris* in Hawaii, where shrimp mortality was reported to be greater than 90% (Lightner et al., 1983). IHHNV is an icosahedral, non-enveloped, parvovirus with a diametric size of 22 nm. The IHHNV genome is comprised of single-stranded DNA that encodes three open reading frames (ORFs), which correspond to the non-structural protein, ORF2 and a 37 kDa coat protein (Bonami et al., 1990; Mari et al., 1993). Based on a genomic similarity analysis with other genomes of viruses that affect invertebrates (mosquitoes), IHHNV was assigned to the genus Brevidensovirus (Shike et al., 2000). IHHNV infects several species of penaeid shrimp in Asia, Central America and South America. The rate of *L. vannamei* mortality caused by IHHNV is relatively low. However, the typical manifestation of IHHNV-induced disease includes growth retardation, which is characterized by high levels of energy consumption, and runt deformity syndrome, which is characterized by cuticular deformities and bent rostrum (Kalagayan et al., 1991).

In 2002, infectious myonecrosis virus (IMNV) was identified as the causative agent of muscle disease of *L. vannamei* farmed shrimp in northeast Brazil. The virus spread rapidly to other neighbor regions in Brazil and, by 2003, was responsible for economic losses that valued at approximately US$20 million (OIE, World Animal Health Organization, 2009). In 2007, outbreaks of IMNV in *L. vannamei* farmed in Indonesia were confirmed (Senapin et al., 2007). IMNV is a non-enveloped icosahedral virus, containing double-stranded RNA genome

and has been assigned to the family Totiviridae (Poulos et al., 2006). Indeed, tridimensional image reconstruction of the IMNV virion revealed a120 kDa capsid protein that has a totivirus-like architecture (Tang et al., 2008). The typical disease symptoms caused by IMNV include loss of hepatopancreas volume, loss of transparency and coloration around the tail, necrosis of the abdomen and cephalothorax, white foci in muscle, and progressive necrosis of the tail fan. Morbidity and mortality can occur at any stage, but juvenile shrimps appear most susceptible to moratlities (Lightner et al., 2004). Environmentally-induced stress seems to be the main trigger for both the chronic and acute onset of IMNV disease.

During a routine monitoring program for the presence of IHHNV and IMNV in *L. vannamei* collected from farms located in northeast Brazil, co-infections with both viruses were detected by reverse transcription PCR (RT-PCR) at relatively high prevalence in shrimp samples suspected of viral disease. In addition, as assessed by quantitative real time PCR (qPCR), the absolute differences in the level of IHHNV and IMNV copy numbers in infected shrimps was measured and compared. The reciprocal values for viral load suggested that a competition between the two viruses for the host might be occurring.

2. Materials and methods

2.1. Samples

Juvenile and sub-adult *L. vannamei*, suspected of viral disease, were sampled from 10 shrimp farms located in the states of Ceará and Rio Grande do Norte, approximately 700 km from one another. Biopsied gill tissue was immediately transferred to microtubes containing RNA Later solution (Ambion/Applied Biosystems, Austin, TX-USA) for extraction of total RNA. Samples were maintained at 4 °C until processed within one week following collection.

2.2. Total RNA preparation

Total RNA was extracted from minced gill (10–20 mg) using the SV Total RNA Isolation System (Promega, Madison, WI-USA), according to the manufacturer's protocol. The quality and yield of total RNA were verified by assessing the integrity of 28S and 18S rRNA, using denaturing agarose gel electrophoresis, and by 260/280 nm ratio spectrophotometric assessment.

2.3. Complementary DNA (cDNA) synthesis

For cDNA synthesis, 1 μg each DNase I-treated total RNA sample, mixed with 500 ng random primers (Promega, Madison-WI, USA) in a final volume of 10 μl, was heated to 70 °C for 10 min, and then placed on ice. ImProm-II reverse transcriptase (Promega, Madison, WI-USA) (100 U) was added together with 1 mM each deoxynucleoside triphosphate (dNTP), 2 mM $MgSO_4$, 1 mM DTT, 20 U recombinant RNase inhibitor, and nuclease-free water to a final volume of 20 μl. The reverse transcription mixture was incubated at 42 °C for 1 h, and then at 70 °C for 15 min. The cDNA was diluted 10-fold with TE (10 mMTris–HCl, pH 7.5, 1 mMEDTA) and 2 μl aliquots were used for analysis of viral infection.

2.4. RT-PCR detection of IHHNV and IMNV

To detect both IHHNV and IMNV, total RNA was isolated from the gill of juvenile and sub-adult *L. vannamei* and used for the synthesis of tandem-primed cDNA, which served as template for (RT-) PCR and quantitative (q-) PCR. The PCR primers used were those recommended by the World Organization for Animal Health (OIE): for detection IHHNV, 77012F and 77353R, and for IMNV, 4587F and 4914R. Each PCR (15 μl) contained 1×PCR buffer (60 mM Tris–SO_4, pH 8.4, 18 mM ammonium sulfate, 2.0 mM $MgSO_4$), 1.5 μl shrimp gill cDNA template (~10 ng cDNA), 1 U GoTaq DNA polymerase (Promega, Madison-WI, USA), 2.0 mM $MgCl_2$, 0.2 μM dNTPs, and 0.2 μM of each specific primer. Thermal cycling conditions used were 95 °C for 5 min, 10 cycles at 95 °C for 50 s, 60 °C for 50 s (minus 1 °C per cycle), and 72 °C for 50 s, followed by 25 cycles at 95 °C for 50 s, 50 °C for 50 s, and 72 °C for 50 s, and a final step at 72 °C for 8 min. A 7.5 μl aliquot of each PCR separated by electrophoresis in a 1.5% agarose gel and DNA was detected by ethidium bromide staining and visualization using a UV transilluminator.

2.5. Absolute quantitative real time PCR of IHHNV and IMNV

For the quantification of IHHNV and IMNV load in *L. vannamei*, the absolute quantitative strategy was used. The gene encoding the nonstructural proteins of IHHNV (GenBank accession number AAF59415.1) and of IMNV (GenBank accession number AAT67231.1) were cloned and serial 10-fold dilution of each gene was made to establish qPCR standard curves. The standard curve series were made in triplicates. Primers for qPCR

detection of IHHNV were 77012F and 77353R (amplicon size 341 nt), MV1125F and MV1469R (amplicon size 344 nt) for IMNV, and βActF (5′-CCACGAGACCACCTACAAC-3′) and βActR (5′-GAAGCACTTCCTGTGAACAAT-3′) (amplicon size 183 nt). Amplification of IHHNV and IMNV non-structural protein cDNAs were carried out in the Rotor-Gene 3000 operated with its respective software, version 6.0.19 (Corbett Research, Mortlake, Australia). Each reaction, in a final reaction volume of 25 µl, consisted of 2.0 µl cDNA aliquots (~10 ng of reverse transcribed mRNA), 200 nM each gene specific sense and anti-sense primers, 12.5 µl GoTaq qPCR Master Mix (Promega, Madison-WI, USA). Amplification conditions were as follows: 95 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s, in 45 repetitions. Fluorescence was collected at 494 to 521 nm during the extension phase.

2.6. Data analysis of qPCR

To calculate the copy number in qPCR experiments, the following equation was used (http://www.uri.edu/research/gsc/resources/cndna.html): number of copies=[DNA amount (ng) * 6.022×10^{23}] / [DNA length (nt)*1×10^{9}*650]. Thus, for example, 1 µg of 1131bp shrimp β-actin cDNA=8.19×10^{11} molecules. Threshold and threshold cycle (Ct) values were automatically determined by Rotor Gene 6.0.19 software, using default parameters. Based on Ct values, the copy number of molecules was calculated according to the following equations: β-actin copy nr=$10^{(-0,268*Ct+9,907)}$ (R=0.9999; R^2=0,9998); IHHNV copy nr=$10^{(-0,249*Ct+11,545)}$ (R=0.9992; R^2=0,9985); IMNV copy nr=$10^{(-0,281*Ct+10,948)}$ (R=0.9997; R^2=0,9995). All measurements were obtained as the mean of at least nine measurements ± SEM (less than 5% error). The corresponding real-time PCR efficiencies (E) of cycles in exponential phase were calculated from the given slopes (k) in Rotor Gene 6.0.19 software, according to the equation: $E=10^{(-1/k)}-1$. The linearity was expressed as square of Pearson correlation coefficient (R^2). For normalization of the values of IHHNV and IMNV load, the mean copy number of β-actin transcripts in each sample, equivalent to 1 µg, were determined from at least 10 independent experiments (n>20), and the results of viral infection expressed as logarithm of copy number. Statistical analysis was conducted with BioStat 5.0 software (Ayres et al., 2007), by using one-way ANOVA with IHHNV as the fixed condition of viral load. Where significant differences were observed, the Least Significant Differences (LSD) test was applied.

3. Results and discussion

Both IHHNV and IMNV routinely cause episodic annual outbreaks in *L. vannamei* farmed in northeast Brazil and the prevalence data on these two viruses in this region has been previously reported (Lightner et al., 2004; Poulos et al., 2006; Poulos and Lightner, 2006; Braza et al., 2009). Almost 100 shrimp samples suspected of viral disease were examined. These samples were from farms located in two Brazilian states and composed by juvenile and sub-adult shrimps. Recently, we demonstrated that PCR-based diagnosis of IHHNV, based on the shrimp genomic DNA assembly and OIE recommended primers (pair 77012F/77353R, Office International des Epizooties — OIE), was not sufficiently robust to discriminate between active infectious IHHNV and integrated non-infective IHHNV genome (Teixeira et al., 2010). Indeed, by using shrimp genomic DNA for detection of IHHNV, we observed that an unexpected high number of samples scored positive for the presence of this virus. One explanation for this observation might be the presence of IHHNV-related sequence in the genome of penaid shrimps. In fact, it was previously reported that IHHNV-related DNA sequences occur in the genome of *Penaeus monodon* from Africa and Australia. These IHHNV sequences were found to be associated with genomic-integrated non-infective type A and type B forms of IHHNV, and a PCR assay was further developed for discriminating between infectious IHHNV and virus-related sequences (Tang and Lightner, 2006; Tang et al., 2007).

As an alternative strategy to confirm the presence of active IHHNV, and simultaneously detect IMNV—a RNA virus, we based essentially our diagnostic tests on cDNA-based strategy for virus detection. The cDNA constitutes the *in vitro* product of messenger RNAs, which results from the transcription of specific gene products. IHHNV has a single stranded DNA genome, while IMNV has a double stranded RNA genome. To replicate, IHHNV first needs to have its genes effectively transcribed into mRNA. Thus, only an actively replicating virus, which produced mRNA transcripts, will be differentially detected by a RT-PCR. On the other hand, to detect the presence of IMNV, a RNA virus, the shrimp cDNA must unconditionally be first prepared. In our present survey, samples that scored positive for IHHNV and IMNV are shown in Fig. 1, panels A and B.

Indeed, the step of cDNA preparation is an additional requirement for dual analysis of both viruses simultaneously by RT-PCR, but this extra procedure allows for the screening of IMNV infection and investigation for the presence of active IHHNV. By using such cDNA based strategy for IHHNV and IMNV detection, we surprisingly found that a large number of samples were positive for both (IHHNV and IMNV) viruses (Fig. 1and Table 1).

Interestingly, as seen also in Fig. 1 (panels A and B), the co-infection was accompanied by differences in the amount of viruses (IHHNV or IMNV) that were detected. This was particularly noticeable because the same quantity of nucleic acid template (here, cDNA) was analyzed, but distinct intensity of each PCR band in the agarose gel was observed. So, we decide to quantify the difference of IHHNV and IMNV load by absolute qPCR. In fact, the analysis by qPCR of dozens of shrimp samples, including some presented in Fig. 1, demonstrated the occurrence of reciprocal presence of each virus in the same sample, that is, in a given sample when the number of IHHNV is higher, the number of IMNV is lower and vice-versa (Fig. 2).

The difference in the amount of each virus in particular samples was statistically significant ($p<0.0001$) and might indicate that one type of virus impairs the replication of the other, often to the detriment of its own viral replication. However, this hypothesis needs further investigation.

Reverse transcription coupled to the polymerase chain reaction (RT-PCR) has also served to confirm the presence of an association between the *Macrobrachium rosenbergii* nodavirus (MrNV) with the extra small virus (XSV), which are both epizootic agents involved in white tail disease (WTD) in the giant freshwater prawn (*M. rosenbergii*), in hatcheries and nursery ponds located in Taiwan (Wang et al., 2007). In fact, viral co-infection is not unusual in farmed animals, and such phenomena have been observed, for example, in pigs (Fraile et al., 2009; Jung et al., 2009) and chickens (Seifi et al., 2009). In invertebrates, Kanthong and collaborators (2010) have shown that persistent triple-virus co-infections could be obtained by successive challenges of mosquito cell cultures with Dengue virus (DEN), insect parvovirus (densovirus) (DNV), and Japanese encephalitis virus (JE). In shrimp, simultaneous infection caused by hepatopancreatic parvovirus (HPV), monodon baculovirus (MBV), and white spot syndrome virus (WSSV), was also previously detected in *P. monodon* post-larvae from a hatchery in India (Manivannan et al., 2002). Prevalent double co-infection of *L. vannamei* with IHHNV and Taura syndrome virus (TSV), and even triple-virus infection with IHHNV, TSV and WSSV, has been found among samples in distinct shrimp farms located in Hainan Province, in China (Tan et al., 2009).

Real-time PCR has been used for absolute and relative quantification of nucleic acid template molecules (DNA and RNA) in a variety of applications, which include pathogen detection and loads, gene expression analysis, single nucleotide polymorphism and genotyping (Wilhelm & Pingoud, 2003). In recent years, qPCR has been used to count the number of *Vibrio parahaemolyticus* in natural shrimp samples (Robert-Pillot et al., 2010), to

detect genotypes of yellow head virus (YHV) that usually infect *P. monodon* (Wijegoonawardane et al., 2010), and to detect IHHNV (Yue et al., 2006), to mention a few examples.

Our present work, however, is the first to report the occurrence of IHHNV and IMNV co-infection in *L. vannamei* farmed in northeast Brazil, and quantitatively discriminate the viral load in such circumstances. Certainly, the coupled strategy we have used to detect the prevalence of double-virus co-infections of shrimp with IHHNV and IMNV in farmed *L vannamei* will allow technical personnel to distinguish between infectious and non-infections IHHNV in shrimp, including broodstock. In addition, RT-PCR can be readily employed in the routine diagnostic screening program for shrimp viruses in the aquaculture industry. Moreover, in combination with qPCR, diagnosis of the viral load and co-infection can be absolutely assessed and the data used to assist differential management plans for epizootic control.

Acknowledgments

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References

Ayres, M., Ayres Jr, M., Ayres, D.L., Santos, A.A.S. 2007. BioEstat: aplicações estatísticas nas áreas das ciências biológicas e médicas. Belém; Sociedade Civil Mamirauá: MCT-CNPq, 364p.

Bonami, J.R., Trumper, B., Mari, J., Brehelin, M., Lightner, D.V., 1990. Purification and characterization of the infectious hypodermal and haematopoietic necrosis virus of penaeid shrimps. J. Gen. Virol. 71, 2657–2664.

Braza, R.F.S., Oliveira da Silva, C.P.R., Reisa, L.G., Martins, P.C.C., Sales, M.P., Meissner, R.V., 2009. Prevalence of infectious hypodermal and hematopoietic necrosis virus (IHHNV) in *Penaeus vannamei* cultured in northeastern Brazil. Aquaculture 288, 143–146.

Brock, J.A., Bullis, R., 2001. Disease prevention and control for gametes and embryos of fish and marine shrimp. Aquaculture 197, 137–159.

FAO, 2010. Food and Agriculture Organization of the United Nations. Fisheries and Aquaculture Department. URL: http://www.fao.org/fishery/statistics/en.

Fraile, L., Calsamiglia, M., Mateu, E., Espinal, A., Cuxart, A., Seminati, C., Martín, M., Domingo, M., Segalés, J., 2009. Prevalence of infection with porcine circovirus-2 (PCV-2) and porcine reproductive and respiratory syndrome virus (PRRSV) in an integrated swine production system experiencing postweaning multisystemic wasting syndrome. Can. J. Vet. Res. 73, 308–312.

Jung, K., Renukaradhya, G.J., Alekseev, K.P., Fang, Y., Tang, Y., Saif, L.J., 2009. Porcine reproductive and respiratory syndrome virus modifies innate immunity and alters disease outcome in pigs subsequently infected with porcine respiratory coronavirus: implications for respiratory viral co-infections. J. Gen. Virol. 90, 2713–2723.

Kalagayan, G., Godin, D., Kanna, R., Hagino, G., Sweeney, J., Wyban, J., Brock, J., 1991. IHHN virus as an etiological factor in runt-deformity syndrome of juvenile *Penaeus vannamei* cultured in Hawaii. J. World Aquac. Soc 22, 235–243.

Kanthong, N., Khemnu, N., Pattanakitsakul, S.N., Malasit, P., Flegel, T.W., 2010. Persistent, triple-virus co-infections in mosquito cells. BMC Microbiol. 10, 14-.

Lightner, D.V., 2003. Exclusion of specific pathogens for disease prevention in a penaeid shrimp biosecurity program. In: Lee, C.-S., O'Bryen, P.J. (Eds.), Biosecurity in Aquaculture Production Systems: Exclusion of Pathogens and Other Undesirables. The World Aquaculture Society, Baton Rouge, Louisiana, pp. 81–116.

Lightner, D.V., Redman, R.M., 1998. Strategies for the control of viral diseases of shrimp in the Americas. Fish Pathol. 33, 165–180.

Lightner, D.V., Redman, R.M., Bell, T.A., 1983. Infectious hypodermal and hematopoietic necrosis, a newly recognized virus disease of penaeid shrimp. J. Invertebr. Pathol. 42, 62–70.

Lightner, D.V., Redman, R.M., Poulos, B.T., Nunan, L.M., Mari, J.L., Hasson, K.W., 1997. Risk of spread of penaeid shrimp viruses in the Americas by the international movement of live and frozen shrimp. Rev. Sci. Tech. 16, 146–160.

Lightner, D.V., Pantoja, C.R., Poulos, B.T., Tang, K.F.J., Redman, R.M., Pasos de Andrade, T., Bonami, J.R., 2004. Infectious myonecrosis: new disease in Pacific white shrimp. Glob. Aquac. Advocate 7, 85.

Manivannan, S., Otta, S.K., Karunasagar, I., Karunasagar, I., 2002. Multiple viral infection in *Penaeus monodon* shrimp postlarvae in an Indian hatchery. Dis. Aquat. Organ. 48, 233–236.

Mari, J., Bonami, J.R., Lightner, D., 1993. Partial cloning of the genome of infectious hypodermal and haematopoietic necrosis virus, an unusual parvovirus pathogenic for penaeid shrimps; diagnosis of the disease using a specific probe. J. Gen. Virol. 74, 2637–2643.

OIE, 2009. Manual of Diagnostic Tests for Aquatic Animal Diseases6th Edition. Office International des Epizooties (OIE), Paris, France. 96-104.

Poulos, B.T., Lightner, D.V., 2006. Detection of infectious myonecrosis virus (IMNV) of penaeid shrimp by reverse-transcriptase polymerase chain reaction (RT-PCR). Dis. Aquat. Organ. 73, 69–72.

Poulos, B.T., Tang, K.F.J., Pantoja, C.R., Bonami, J.R., Lightner, D.V., 2006. Purification and characterization of infectious myonecrosis virus of penaeid shrimp. J. Gen. Virol. 87, 987–996.

Robert-Pillot, A., Copin, S., Gay, M., Malle, P., Quilici, M.L., 2010. Total and pathogenic *Vibrio parahaemolyticus* in shrimp: fast and reliable quantification by real-time PCR. Int. J. Food Microbiol. 143, 190–197.

Seifi, S., Asasi, K., Mohammadi, A., 2009. A study of natural co-infection caused by avian influenza (H9 subtype) and infection bronchitis viruses in broiler chicken farms showing respiratory signs. OJVRTM Online J. Vet. Res. 13, 53–62.

Senapin, S., Phewsaiya, K., Briggs, M., Flegel, T.W., 2007. Outbreaks of infectious myonecrosis virus (IMNV) in Indonesia confirmed by genome sequencing and use of an alternative RT-PCR detection method. Aquaculture 226, 32–38.

Shike, H., Dhar, A.K., Burns, J.C., Shimizu, C., Jousset, F.X., Klimpel, K.R., Bergoin, M., 2000. Infectious hypodermal and hematopoietic necrosis virus of shrimp is related to mosquito brevidensoviruses. Virology 277, 167–177.

Tan, Y., Xing, Y., Zhang, H., Feng, Y., Zhou, Y., Shi, Z.L., 2009. Molecular detection of three shrimp viruses and genetic variation of white spot syndrome virus in Hainan Province, China, in 2007. J. Fish Dis. 32, 777–784.

Tang, K.F., Lightner, D.V., 2006. Infectious hypodermal and hematopoietic necrosis virus (IHHNV)-related sequences in the genome of the black tiger prawn *Penaeus monodon* from Africa and Australia. Virus Res. 118, 185–191.

Tang, K.F., Navarro, S.A., Lightner, D.V., 2007. PCR assay for discriminating between infectious hypodermal and hematopoietic necrosis virus (IHHNV) and virus-related sequences in the genome of *Penaeus monodon*. Dis. Aquat. Organ. 74, 165–170.

Tang, J., Ochoa, W.F., Sinkovits, R.S., Poulos, B.T., Ghabrial, S.A., Lightner, D.V., Baker, T.S., Nibert, M.L., 2008. Infectious myonecrosis virus has a totivirus-like, 120-subunit capsid, butwithfiber complexes at thefivefoldaxes. Proc. Natl Acad. Sci.USA 105, 17526–17531.

Teixeira, M.A., Cruz, J.E., Vieira, P.R., Branco, I.R., Costa, F.H., Rádis-Baptista, G., 2010. Differential diagnosis of active hypodermal and hematopoietic necrosis virus based on gene choice and reverse transcription coupled with PCR. Genet.Mol.Res. 9,2025–2031.

Wang, C.S., Chang, J.S., Shih, H.H., Chen, S.N., 2007. RT-PCR amplification and sequence analysis of extra small virus associated with white tail disease of *Macrobrachium rosenbergii* (de Man) cultured in Taiwan. J. Fish Dis. 30, 127–132.

Wijegoonawardane, P.K., Cowley, J.A., Walker, P.J., 2010. A consensus real-time RT-PCR for detection of all genotypic variants of yellow head virus of penaeid shrimp. J Virol Methods. 167, 5–9.

Wilhelm, J., Pingoud, A., 2003. Real-time polymerase chain reaction. Chembiochem 11, 1120–1128.

Yue, Z.Q., Liu, H., Wang, W., Lei, Z.W., Liang, C.Z., Jiang, Y.L., 2006. Development of real time polymerase chain reaction assay with TaqMan probe for the quantitative detection of infectious hypodermal and hematopoietic necrosis virus from shrimp. J. AOAC Int. 89, 240–244.

Table 1 Overall RT-PCR data from *L. vannamei* shrimp suspected of IHHNV and IMNV infection in two states of Northeast Brazil.

Location of sampling	Nr of samples (and%)	Nr of positives[a] (and%) for		
	Tested by RT-PCR	IHHNV infection	IMNV infection	IHHNV+IMNV co-infection
Ceará	30 (100)	11 (36.7)	01 (3.3)	05 (26.7)
Rio Grande do Norte	40 (100)	09 (22.5)	11 (27.5)	20 (50.0)

[a] Only numbers of positive samples are presented in the table. Samples were collected by February 2010. RT-PCR=reverse transcriptase PCR made by using shrimp cDNA as template; IHHNV+IMNV=observed natural co-infection with both virus.

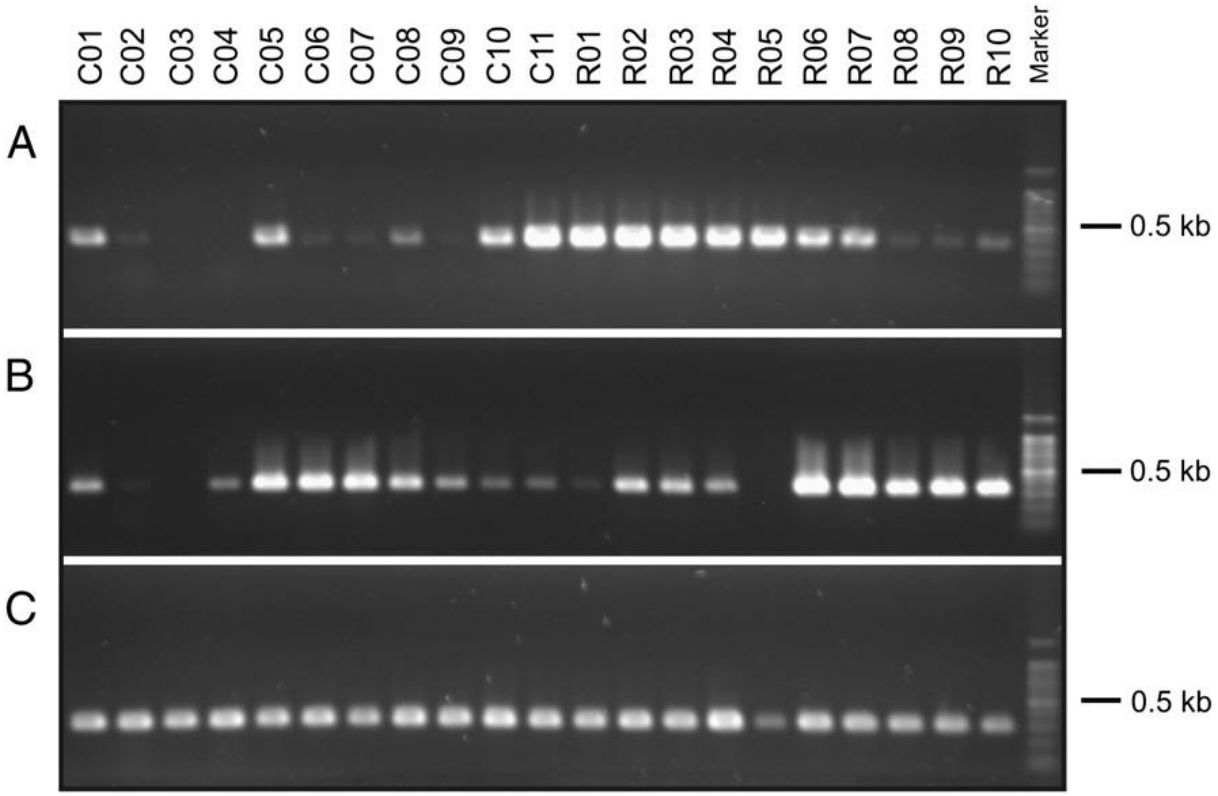

Fig. 1. Reverse transcription coupled to PCR (RT-PCR)-based diagnosis for the detection of an active infection with IHHNV and co-infection with IMNV in *L. vannamei*. In this test, the shrimp complementary DNA (cDNA) was used as a template. Panel A: representative samples that are positive for IHHNV (as illustrated by the product of the amplification reaction with the primer pair 77012 F/77353R). Panel B: the same samples that are shown in panel A, however, the samples were analyzed for IMNV infection using the OIE recommended primers 4587 F and 4914R. In panel C, the product of the amplification reaction of the penaeid β-actin gene that was used as internal control. Lanes in each individual position, in all panels, correspond to the same sample.

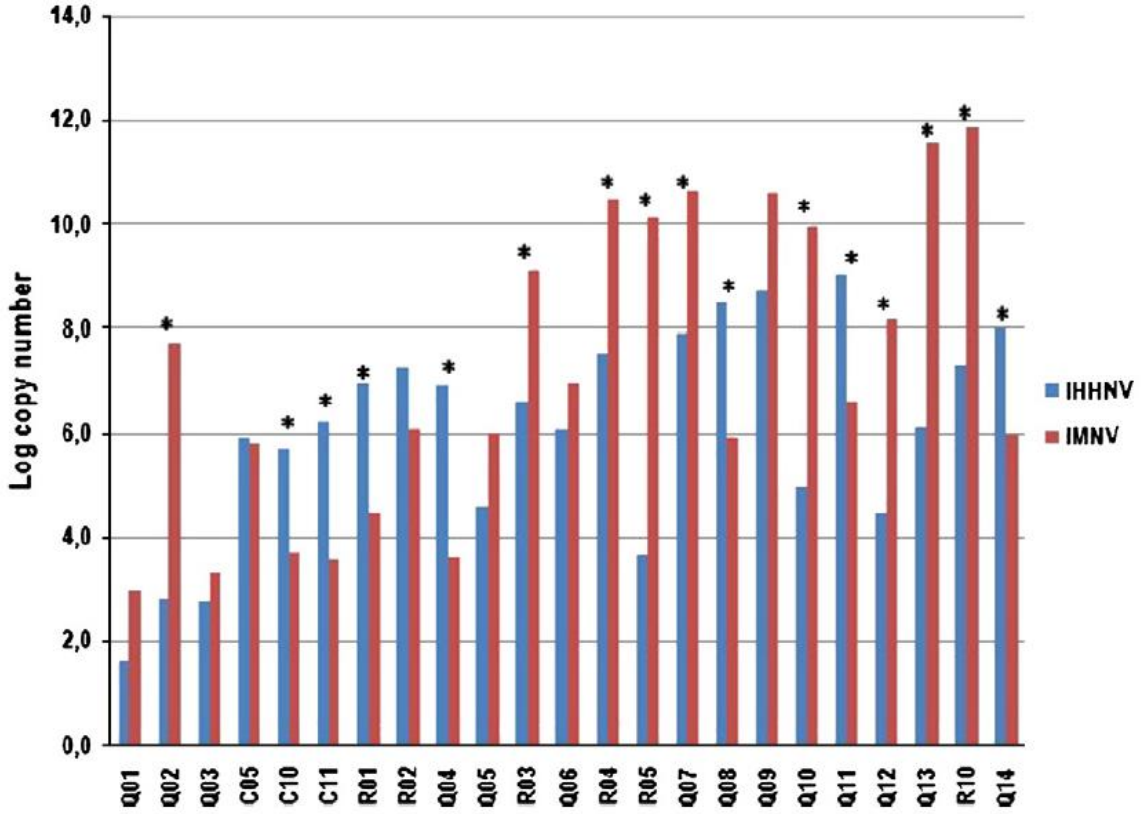

Fig. 2. Logarithm of mean copy number of IHHNV and IMNV co-infecting farmed *L. vannamei*. Non-structural protein cDNAs of IHHNV and IMNV were quantified in each shrimp sample (n>6) by absolute qPCR, then normalized in function of 1 μg of β-actin transcripts, and the logarithm (Log_{10}) of copy number calculated accordingly. Asterisks denote differences that are statistically significant (P<0.0001).

7 CAPÍTULO II

Differential induction of HSP-70 expression in response to IHHNV in white shrimp *Litopenaeus vannamei* naturally co-infected with IHHNV and IMNV

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Abstract

Brazil is becoming one of the main global producers of the shrimp *Litopenaeus vannamei*. Worldwide outbreaks of viral disease place this aquaculture industry at risk, causing episodic economical loss. The primary viruses for *L. vannamei*, particularly in northeastern Brazil, are the infectious hypodermal and hematopoietic necrosis virus (IHHNV) and the infectious myonecrosis virus (IMNV). After a period of unusual rainfall, we detected that farmed shrimp developing IMN or IHHN disease were co-infected with both viruses, and the disease outcome resulted from reciprocal IHHNV and IMNV proliferation. To comprehend how the key molecules of innate immunity respond to this double infection, the levels of HSP-70, crustin, penaeidin-3a, and C-type lectin-br1 were assessed by quantitative PCR. HSP-70 expression was expressively up-regulated by IHHNV infection in the gills of double-infected shrimp but not by IMNV infection; the other transcripts were not significantly altered. These findings implicate the HSP-70 as a differential modulator of viral co-infection in shrimp.

Keywords: Shrimp virus, IHHNV, IMNV, co-infection, qPCR, innate immunity gene.

Background

Penaeid shrimp aquaculture is a crescent global industry that is valued in billions of US dollars but is affected episodically by bacterial and viral diseases that cause significant economical losses (Lightner and Redman 1998; Lightner 2003; Flegel 2006). In Brazil, two viruses are the primary causative agents of epizootic diseases of *Litopenaeus vannamei* farmed in the northeast of Brazil: infectious hypodermal and hematopoietic necrosis virus (IHHNV) and infectious myonecrosis virus (IMNV). IHHNV is a singlestranded DNA virus that, upon infection, causes symptoms such as shrimp deformities and growth retardation (Lightner et al. 1983). In contrast, IMNV is a non-enveloped, double-stranded RNA virus that causes severe muscle degeneration and high rates of mortality in affected shrimp (Lightner et al. 2004). In previous work, we reported the occurrence of the natural co-infection of L. vannamei (the Pacific whiteleg shrimp) with IHHNV and IMNV in Brazil (Teixeira-Lopes et al. 2011). In that report, we showed that the most positive samples of shrimp were simultaneously co-infected with both viruses, but the disease symptoms and outcome, as seen in the field, indicated that one type of virus might be predominating and impairing the replication of the other, often to the detriment of its own viral replication.

L. vannamei is a decapod that relies on molecules of the innate immune system to defend itself from microorganisms (Iwanaga and Lee 2005). Essentially, the innate invertebrate immune system is based on cellular and molecular components that, upon infection, recognize molecular patterns in the microbial membranes and contribute to the triggering of a cascade of events that culminate with the killing of pathogens. For example, C-type lectins, calcium-dependent proteins with one or more conserved carbohydrate recognition domains (CRDs), are known to act as pattern recognition receptors by binding to pathogen-associated molecular patterns and activating the innate host defense (Fujita et al. 2004). C-type lectins and C-type lectin-like domaincontaining proteins have been identified in several species of crustaceans, including crabs (Kong et al. 2008) and shrimp (Luo et al. 2006; Liu et al. 2007; Costa et al. 2011), as well as in other invertebrates such as barnacles (Matsubara et al. 2007), cnidarians (Wood-Charlson and Weis 2009), and mollusks (Yamaura et al. 2008; Zhang et al. 2009). Other polypeptides that are conjunctly involved in innate defense in penaeid shrimp include members of the family of antimicrobial peptides and heat shock proteins. Crustins and penaeidins, for example, are two classes of antimicrobial peptides that are expressed in crustacean and shrimp tissues. Initially identified in the crab Carcinus maemas, crustins were collectively named carcinines (Schnapp et al. 1996). In *L.*

vannamei and *Litopenaeus setiferus*, more than one isoform precursor has been found (Gross et al. 2001). Crustins are categorized into three types (I to III) according to their functions and the arrangement of their protein domains (Smith et al. 2008). In the epipodite, but not the hemocytes, of the penaeid shrimp *Penaeus monodon*, crustinlike molecules were observed to be up-regulated upon heat treatment and hyperosmotic salinity stress. This up-regulation revealed the crustin-like molecules' function as a stress mediator in addition to their in vitro antibacterial action against gram-positive bacteria (Vatanavicharn et al. 2009). The family of penaeidins also includes members with variable amino acid sequences and functions. Three families that show biological activity against filamentous fungi and bacteria have been characterized from the hemolymp of *L. vannamei* (Destoumieux et al. 1997). The expression pattern of penaeidins-2, -3, and −4 in the hemocytes of L. vannamei was studied by relative (qPCR) and was shown to fluctuate; penaeidin-3 was expressed 103-fold more than penaeidin-2 and penaeidin-4 were (O'Leary and Gross 2006). Another class of polypeptides that are recruited in response to an elicitor and are connected to the innate immune system of crustacean encompasses the heat shock proteins (HSPs). The HSPs are intrinsic chaperones that include polypeptides with molecular weights in the range of 16 to 100 kDa, which correspond to their family names (e.g., HSP21, HSP-60, HSP-70, and HSP-90), that are expressed by all cellular organisms in response to a stress (Roberts et al. 2010). In *L. vannamei*, the expression profiles of HSP60 and HSP-70 in response to bacterial infection and heat shock have been studied and reveal that both proteins are significantly up-regulated in the tissues of the gill, hepatopancreas, and hemocytes after shrimp exposure to bacteria, such as *Staphylococcus aureus* and *Vibrio alginolyticus* (Zhou et al. 2010).

Due to the biological roles that these families of molecules play in the innate immunity of most invertebrates, and particularly that of *L. vannamei*, they are adequate molecular markers to deeper investigate their pattern of expression in response to epizootics. In the field, farmed shrimp are subjected to several simultaneous stress stimuli such as changes in temperature, osmotic balance, feeding scheme, and bacterial and viral assaults. Consequently, the induction of gene expression, and ultimately the control or susceptibility of an infectious disease, is the result of an intricate balance of these biological and environmental factors. Moreover, because most experiments that involve expression profiling are conducted in the laboratory, under strict controlled conditions, we decided to investigate how representative members of the four classes of genes connected to the immune response (crustin, C-type lectin, HSP, and penaeidin) oscillate in farmed shrimp that are naturally infected with IMNV and IHHNV. We have demonstrated that the level of HSP-70 expression in co-infected

farmed shrimp is up-regulated in response to IHHNV proliferation, but it seems unrelated to the IMNV particle number.

Methods

Shrimp samples

Sub-adult *L. vannamei* (average body weight 10.16 ± 4.19 g), growing at a density of 40 to 60 individuals per m^2 in a temperature range from 26°C to 30°C, and a salinity of 10 to 40 ppt, were sampled from local shrimp farms (in the states of Ceará and Rio Grande do Norte) and classified as asymptomatic, IHHNV- or IMNV-infected shrimp based on the visual inspection of a gross signal of disease. IHHN-affected shrimp show growth retardation, which is characterized by high levels of energy consumption, and runt deformity syndrome, which is characterized by cuticular deformities and a bent rostrum (Kalagayan et al. 1991). Shrimp suffering from IMN disease show a loss of transparency and coloration around the tail, necrosis of the abdomen and cephalothorax, white foci in the muscle, progressive necrosis of the tail fan, and high rates of mortalities. The gill tissue from the individual samples was biopsied and immediately transferred to microtubes containing RNAlater solution (Ambion/Applied Biosystems, Austin, TX, USA) for the preservation of total RNA. The samples were maintained at 4°C until they were processed within 1 week following collection.

Total RNA preparation

Total RNA was extracted from minced gills (10 to 20 mg) using the SV Total RNA Isolation System (Promega, Madison, WI, USA), according to the manufacturer's protocol, which includes a step of DNaseI treatment. The quality and yield of total RNA were verified by assessing the integrity of 28S and 18S rRNA by formamide-based denaturing agarose gel electrophoresis (Masek et al. 2005) and by 260/280 nm ratio spectrophotometric assessment.

Complementary DNA (cDNA) synthesis

For complementary DNA (cDNA) synthesis, 1 μg of each DNase I-treated total RNA sample, mixed with 500 ng of random primers (Promega, Madison, WI, USA) in a final

volume of 10 μl, was heated to 70°C for 10 min and then placed on ice. ImProm-II reverse transcriptase (Promega, Madison, WI USA) (100 U) was added together with 1 mM of each deoxynucleoside triphosphate, 2 mM $MgSO_4$, 1 mM dithiothreitol, 20 U recombinant RNase inhibitor, and nuclease-free water to a final volume of 20 μl. The reverse transcription mixture was incubated at 42°C for 1 h and then at 70°C for 15 min. The cDNA was diluted tenfold with TE (10 mM Tris–HCl, pH 7.5, 1 mM ethylenediaminetetraacetic acid), and 2 μl aliquots were used for relative and quantitative real time PCR (qRT-PCR) experiments.

Absolute quantitative real time PCR

For the quantification of IHHNV and IMNV load in *L. vannamei* and to determine the expression profile of the innate immunity genes, the absolute and relative quantitative strategies were used. The genes encoding the non-structural proteins of IHHNV and of IMNV, as well as *L. vannamei* β-actin, crustin, C-type lectin, penaeidin-3a, and HSP-70 (Table 1), were cloned into pGEM T-easy (Promega, Madison, WI, USA), and serial tenfold dilutions of each gene were made to establish the quantitative PCR (qPCR) standard curves. The standard curve series were made in triplicate. The primers used for qRT-PCR assessment of the viral load and the expression profile analysis of innate defense gene transcripts are listed in Table 1.

The amplification of virus (IHHNV and IMNV) and of *L. vannamei* (β-actin, crustin C-type lectin, penaeidin-3a, and HSP-70) cDNAs was carried out in the Rotor-Gene 3000 that was operated with its respective software, version 6.0.19 (Corbett Research, Mortlake, Australia). Each reaction, in a final reaction volume of 25 μl, consisted of 2.0 μl cDNA aliquots (approximately 10 ng of reverse transcribed mRNA), 200 nM of each gene specific sense and anti-sense primer, and 12.5 μl GoTaq qPCR Master Mix (Promega, Madison, WI, USA). The amplification conditions were as follows: 95°C for 30 s, 50°C for 30 s, and 72°C for 30 s, in 45 repetitions. Fluorescence was detected at 494 to 521 nm during the extension phase.

Data analysis of qRT-PCR

To calculate the copy number in the absolute qPCR experiments, the following equation was used (http://www.uri.edu/research/gsc/resources/cndna.html): Number of copies = [DNA amount (ng) * 6.022×10^{23}] / [DNA length (nt) * 1×10^{9} * 650]. The threshold and

threshold cycle values were automatically determined by the Rotor Gene 6.0.19 software, using the default parameters. All measurements were obtained as the mean of at least nine measurements ± SEM (less than 5% error). The corresponding real-time PCR efficiencies (E) of the cycles in the exponential phase were calculated from the given slopes (k) in the Rotor Gene 6.0.19 software, according to the equation $E = 10^{(-1/k)} - 1$. For normalization of the values of the viral load and the innate immunity gene expression, the mean copy number of β-actin transcripts in each sample, equivalent to 1 μg, was determined from at least ten independent experiments ($n \approx 30$); the results of the viral infection and gene expression analysis were indicated as the logarithm of the copy number. Statistical analysis was conducted with the BioStat 5.0 software (Ayres et al. 2007). The linearity of the qPCR standard curve was expressed as the square of the Pearson correlation coefficient (R^2); the positive correlation between the viral load and the gene expression level was expressed as the Pearson coefficient (r). Tests of significance were performed with a one-way ANOVA, Tukey test, and the significant difference was considered as $P < 0.01$.

Results

Natural co-infection in farmed *L. vannamei* shrimp with IHHNV and IMNV

A total of 120 shrimp were inspected for a gross signal of viral disease and were classified as asymptomatic, IHHNV- or IMNV-infected, with a total of 50 (approximately 42%), 40 (approximately 33%), and 30 (25%) samples, respectively (Table 2). Through conventional PCR analysis, 98% of the asymptomatic samples scored positive for IHHNV when shrimp genomic DNA from gill was used as the template, and the recommended 'Office International des Epizooties (OIE)' oligonucleotides (77012F and 77353R, amplicon size 341 nt) were used as the primers in the diagnostic reactions. Shrimp samples suspected of IHHNV and IMNV infections were analyzed by reverse transcription coupled with PCR (RT-PCR) with shrimp gill cDNA as the template and oligonucleotides specific for viral genes as the primers (Table 1). RT-PCR analysis revealed that 50% of the samples suspected of IHHNV infection scored positive for IHHNV, 12.5% scored positive for IMNV, and 37.5% were shown to be positive for both IHHNV and IMNV. In case of samples suspected of IMNV infection, 10% scored positive for IHHNV, 6.7% scored positive for IMNV, and 75% scored positive for double-infection with IHHNV and IMNV (Table 2). Molecular diagnostic

analysis also revealed qualitative differences in the ratios of viral load in co-infected shrimp samples (Figure 1). The quantitative differences in IHHNV and IMNV copy number were ascertained by qPCR and confirmed the relationship of the inverse proportionality in the viral loads (next section).

Reciprocal IHHNV and IMNV loads in co-infect shrimp

In the samples of farmed shrimp from Brazil, absolute quantitative real time PCR has shown the reciprocal presence of IHHNV and IMNV; when the number of IHHNV is higher, the number of IMNV is lower and vice-versa (Teixeira-Lopes et al. 2011). The proportionality or reciprocity in IHHNV and IMNV load is seen in Figure 2. The presence of other viruses (yellow head virus (YHV), Taura syndrome virus (TSV), and white spot syndrome virus (WSSV)) and bacteria (necrotizing hepatopancreatic bacteria and Vibrio sp.) was tested, according to OIE recommendations (2011), but was not detected, which confirmed the status of a double-infection with IHHNV and IMNV. In several cases, the ratio IHHNV/IMNV was seen to be approximately 1 when the viral load of both viruses was proportional, more than 1 when the IHHNV load was higher than the IMNV load, and less than 1 when the IHHNV load was inferior to the IMNV load.

The up-regulation HSP-70 is mediated by IHHNV in double-infected *L. vannamei*

In the double-infected shrimp samples, the expression of HSP-70 in the gills is induced by 3 orders of magnitude when IHHNV is the predominant epizootic, which reveals a relatively strong positive correlation ($p < 0.0001$ and $r = 0.7370$) between the number of IHHNV particles and the increase in HSP-70 copy number (Figure 3A). In the case of IMNV, the relationship between the viral load and the expression of the HSP-70 gene was not relevant and is numerically corroborated by its respective statistical values of Pearson correlation and confidence ($p = 0.4777$, $r = 0.1558$) (Figure 3B). The induction of HSP-70 expression in relation to IHHNV infection was also observed in some shrimp samples in which IHHNV was the only infecting virus (Figure 4). In Figure 4, the lack of a relationship between the up-regulation of HSP-70 and the IMNV load is illustrated. The expression of crustin, C-type lectin (CTL-br1), and penaeidin-3a in natural double-infected farmed shrimp was concomitantly evaluated, but in shrimp gill, these genes were not strictly related to either the IHHNV or IMNV load, as seen in Figure 3C,D,E,F,G,H. The absence of a direct

relationship between the induction of the tested innate immune genes (crustin, CTL-br1, and penaeidin-3a) mediated by IHHNV and IMNV alone or by both viruses together in chronically infected shrimp was therefore statistically determined (Figure 3C,D,E,F,G,H and Figure 4).

Discussion

In this study, we investigate the pattern of expression of four representative members of the polypeptide families that are related to the innate immune response of the Pacific whiteleg shrimp *L. vannamei*, in the standard natural condition of raring in farms located in the northeast of Brazil. Brazil is becoming one of the main global producers of this shrimp species, and approximately 98% of the national aquaculture industry is concentrated in the northeastern region of the country. This Brazilian region favors shrimp and fish aquaculture due to the particular climate, which is characterized by low temperature fluctuation and low rainfall throughout the year. Despite these excellent environmental conditions for shrimp farming, an anomalous period of excessive rain occurred in the interval from December 2009 to March 2010. Consequently, a virus outbreak began, likely as a consequence of reduced water salinity and osmotic shock that compromised the regional shrimp production in that period. The samples for molecular diagnostic analysis were collected in these circumstances and were inspected for known symptoms of viral and bacterial disease. Based on the distinct pathologial signs seen in the shrimp infected with IHHNV and IMNV, the two prevalent viruses in Northeastern Brazil, the samples were classified as asymptomatic, IHHNV- or IMNV-suspected (Table 2). Conventional PCR-based analysis proved that 98% of asymptomatic shrimps were IHHNV carriers, although the gross signals of infection were not manifested when they were sampled. The presence of IMNV in asymptomatic shrimp was not tested in such samples because they were collected in a preservation solution for genomic DNA extraction, and IMNV is an RNA virus. Whether carried IHHNV was in its infective form or silently integrated in the shrimp genome, as previously reported (Tang and Lightner 2006; Teixeira et al. 2010), was not addressed in the present study. However, shrimp samples that were grouped and labeled as IHHNV- and IMNV-suspected were analyzed by RT-PCR to confirm the gross signals of chronic viral disease. Unexpectedly, a relatively high number of shrimp samples were co-infected with both viruses, and the disease outcome was the result of the predominance of one kind of virus that quantitatively proliferates to the detriment of the other (Figures 1 and 2). In fact, the prevalent co-infection of *L. vannamei* with IHHNV and

other virus, like TSV, and even in association with two other viruses (TSV and WSSV), i.e., triple-virus infection, has been found among samples in distinct shrimp farms located in China (Tan et al. 2009). Therefore, IHHNV seems to be a constant co-infecting agent in shrimp that is capable of genome integration events and of being carried by asymptomatic individuals in all phases of shrimp development.

To understand the immune response of *L. vannamei* chronically co-infected with IHHNV and IMNV in the natural condition of farming, we chose to test four representative classes of polypeptides that are responsible for the first line of defense against pathogenic microorganisms. These molecular markers of the innate immune response include two classes of antimicrobial peptides (crustin and penaeidin), a C-type lectin and a heat shock protein (HSP-70). qPCR was used to evaluate the expression patterns of the transcripts that corresponded to the four polypeptides. The analysis was focused on the gill tissue because this shrimp organ is the first to accumulate pathogenic microorganisms (Burgents et al. 2005), and several studies have reported the differential gene expression related to environmental stress in the gill (Zhou et al. 2010; Wang et al. 2011).

As was observed by means of experimental results with samples that were environmentally exposed and thriving with IHHNV and IMNV co-infection (Figure 3A), the higher the number of IHHNV particles, the higher the level of HSP-70 expression in the shrimp gill, and values reached at least 3 orders of magnitude in comparison with estimates from the basal level of HSP-70 copy number. On the contrary, the proliferation of IMNV in co-infect shrimp neither caused such a proportional increase in the HSP-70 level as that which was observed for IHHNV nor caused a down-regulation of this gene, as statistically tested (Figure 3B). The validation of such an observation is also applicable for samples in which IHHNV or IMNV is the single infecting virus (see Figure 4).

The expression levels of crustin, penaeidin and C-type lectin was assessed in the condition of reciprocal viral co-infection. However, the relationship between IHHNV/ IMNV reciprocal co-infection and crustin or penaeidin expression was not observed, which showed a weak positive correlation (Figure 3C,D,E,F,G,H). Crustin and penaeidin, which are primarily produced in high level in hemocytes, were shown to be expressed differently in several shrimp tissues, including the gill. Crustin and penaeidin act mainly as antimicrobial agents against bacteria and fungi (Tassanakajon et al. 2011), despite of the reported data about their role as anti-virals (Amparyup et al. 2008).

The recently cloned CTL-br1 cDNA was evaluated for the purpose of establishing a relationship between its expression in response to the simultaneous presence of IHHNV and

IMNV in sub-adult farmed shrimps. Despite some level of induction detected in the gill of sub-chronic IHHNV-infected shrimps (Costa et al. 2011), a correlation was observed in the same tissue of *L. vannamei* co-infected with both IHHNV and IMNV. In fact, homologous C-type lectin gene products were observed to fluctuate positively in *L. vannamei* hemocytes in the first 48 h of WSSV infection, which showed a direct anti-viral activity that is displayed by this kind of molecule (Zhao et al. 2009).

To certify that the oscillation in gene expression is virus-specific and not a generalized response, a number of samples were evaluated for the ratio between IHHNV and IMNV copy number and the pattern of expression (Figure 4). When IHHNV is the only virus or when its number predominates in co-infected samples by a difference of twofold, the same pattern of expression for HSP-70 and a positive relationship are observed. Again, no significant relationship was seen when IHHNV is predominant in respect to IHHNV and IMNV co-infected shrimp, although there was a slight increase in samples with the presence of IHHNV alone. The same analysis conducted for IMNV revealed that when IMNV predominates in IHHNV/IMNV double-infected shrimps, or when IMNV is the only infective virus, the level of HSP-70 is still inferior by 3 orders of magnitude when compared to IHHNV in the same circumstances. However, as seen in a number of samples, the levels of crustin, penaeidin, and CTL-br1 are preferentially increased in the shrimp gill when IMNV is present alone. For now, we are unable to speculate this differential pattern of expression, but we can conclude, based on the present data, that the triggering of the innate immune genes by IHHNV and IMNV seemed to be virus-specific and does not overlap even in a double-infected host. The up regulation of HSP-70 IHHNV might confer a certain degree of immunity in shrimp through activation of the toll-like receptor transduction pathway (Tsan and Gao 2004). Thus, HSP-70 may display a dual role: it exerts immune activation as danger signals, mediates protection against infectious diseases, or exhibits regulatory activities in controlling and preventing disease (Multhoff 2006). In fact, shrimp exposed to sub-lethal doses of virus induce a status of ‘pseudo-vaccination’ of the host, improving the resistance and survival of *Parribacus japonicus* to high infective doses of WSSV (Wu et al. 2002). However, as observed through our data, with excessive increases of HSP-70 expression, damage to the shrimp tissues may occur, due to pro-inflammatory cytokines that are normally produced, which would facilitate IMNV proliferation. In fact, this explanation is plausible because shrimp carrying IHHNV, or in which the number of IHHNV particles is predominant, develop IHHN disease, but not IMN disease. In contrast, shrimp that develop IMN disease are most likely to be co-infected with both IHHNV and IMNV (Table 2).

In summary, to our knowledge, the present study is the first to correlate the up-regulation of the HSP-70 gene mediated by a virus in naturally double-infected farmed shrimp host. The modulation of HSP expression has implications in the activation of the innate immunity response and may serve as a mean of inducing pseudo-vaccination. However, in double infected shrimp, the triggered induction of HSP-70 that would contribute to counteract a pathogen assault seems to open a window of immune-modulator imbalance that might be deleterious to the host due to the presence of an opportunistic co-infecting virus. Obviously, more comprehensive studies are required to elucidate the molecular mechanisms that switch on and off in the operation of reciprocal viral proliferation in shrimp, as well as their roles in the molecular arsenal of the host defenses that are necessary to maintain homeostasis. In fact, to better understand the inductive expression of HSP-70 response to IHHNV in white shrimp (*L. vannamei*) infected with predominant IMNV or only IMNV, we are conducting quantitative expression analysis with samples of shrimps infected in the laboratory under controlled conditions.

Conclusions

The innate immune response comprises a complex of several player molecules that assist the host to neutralize infectious assaults by different microbes. Like other organisms, shrimps also rely their defense on the innate immune components to annihilate bacterial and viral infection. As observed in the present study, in naturally co-infected farmed shrimp, IHHNV triggers the expression of HSP-70, a molecule that might serve to counteract viral infection. Paradoxically, the increase of HSP-70 expression in response to the IHHNV loads seems to facilitate the propagation of IMN virus. In conclusion, we present experimental evidence to show that in farmed shrimps that are environmentally exposed to viral double-infection, a disease outcome might be partially linked to differential fluctuations in HSP-70 expression in the entry port, the shrimp gill.

Abbreviations

IHHNV: infectious hypodermal and hematopoietic necrosis virus; IMNV: infectious myonecrosis virus; CRDs: carbohydrate recognition domains; HSPs: heat shock proteins; qPCR: quantitative real time PCR; RT-PCR: reverse transcriptase coupled with PCR; TSV:

Taura syndrome virus; WSSV: white spot syndrome virus; OIE: Office International des Epizooties; cDNA: complementary DNA.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

FHFC and GRB participated in the research design. PRNVG and GRB conducted the experiments. ÍRCBR and FHFC collected and diagnosed the gross signals of viral infection in shrimp samples from farms. PRNVG and GRB performed the data analysis. PRNVG, FHFC, and GRB wrote or contributed to the writing of the manuscript. All authors read and approved the final manuscript.

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References

Amparyup P, Donpudsa S, Tassanakajon A (2008) Shrimp single WAP domain (SWD)-containing protein exhibits proteinase inhibitory and antimicrobial activities. Dev Comp Immunol 32:1497–1509

Ayres M, Ayres Jr.M, Ayres DL, Santos AAS (2007) BioEstat: aplicações estatísticas nas áreas das ciências biológicas e médicas. Sociedade Civil Mamirauá: MCT-CNPq, Belém

Burgents JE, Burnett LE, Stabb EV, Burnett KG (2005) Localization and bacteriostasis of Vibrio introduced into the Pacific white shrimp, *Litopenaeus vannamei*. Dev Comp Immunol 29:681-691

Costa FH, Valença NS, Silva AR, Bezerra GA, Cavada BS, Rádis-Baptista G (2011) Cloning and molecular modeling of *Litopenaeus vannamei* (Penaeidae) C-type lectin homologs with mutated mannose binding domain-2. Genet Mol Res 10:650-64

Destoumieux D, Bulet P, Loew D, Van Dorsselaer A, Rodriguez J, Bachère E (1997) Penaeidins, a new family of antimicrobial peptides isolated from the shrimp *Penaeus vannamei* (Decapoda). J Biol Chem 272:28398-28406

Flegel TW (2006) Detection of major penaeid shrimp viruses in Asia, a historical perspective with emphasis on Thailand. Aquaculture 258:1-33

Fujita T, Matsushita M, Endo Y (2004) The lectin-complement pathway - its role in innate immunity and evolution. Immunol Rev 198:185-202

Gross PS, Bartlett TC, Browdy CL, Chapman RW, Warr GW (2001) Immune gene discovery by expressed sequence tag analysis of hemocytes and hepatopancreas in the Pacific White Shrimp, *Litopenaeus vannamei*, and the Atlantic White Shrimp, *L. setiferus*. Dev Comp Immunol 25:565-577

Iwanaga S, Lee BL (2005) Recent advances in the innate immunity of invertebrate animals. J Bioch Mol Biol 38:128-150

Kalagayan G, Godin D, Kanna R, Hagino G, Sweeney J, Wyban J, Brock J (1991) IHHN virus as an etiological factor in runt-deformity syndrome of juvenile *Penaeus vannamei* cultured in Hawaii. J World Aquac Soc 22:235–243

Kong HJ, Park EM, Nam BH, Kim YO, Kim WJ, Park HJ, Lee CH, Lee SJ (2008) A C-type lectin like-domain (CTLD)-containing protein (PtLP) from the swimming crab *Portunus trituberculatus*. Fish Shellfish Immunol 25:311-314

Lightner DV, Pantoja CR, Poulos BT, Tang KFJ, Redman RM, Pasos de Andrade T, Bonami JR (2004) Infectious myonecrosis: new disease in Pacific white shrimp. Glob Aquac Advocate 7:85

Lightner DV, Redman RM, Bell TA (1983) Infectious hypodermal and hematopoietic necrosis, a newly recognized virus disease of penaeid shrimp. J Invertebr Pathol 42:62–70

Lightner DV, Redman RM (1998) Strategies for the control of viral diseases of shrimp in the Americas. Fish Pathol 33:165–180

Lightner DV (2003) Exclusion of specific pathogens for disease prevention in a penaeid shrimp biosecurity program. In: Lee C-S, O'Bryen PJ (ed) Biosecurity in Aquaculture Production Systems: Exclusion of Pathogens and Other Undesirables, The World Aquaculture Society, Baton Rouge, Louisiana

Liu YC, Li FH, Dong B, Wang B, Luan W, Zhang XJ, Zhang LS, Xiang JH (2007) Molecular cloning, characterization and expression analysis of a putative C-type lectin (Fclectin) gene in Chinese shrimp *Fenneropenaeus chinensis*. Mol Immunol 44:598-607

Luo T, Yang H, Li F, Zhang X, Xu X (2006) Purification, characterization and cDNA cloning of a novel lipopolysaccharide-binding lectin from the shrimp *Penaeus monodon*. Dev Comp Immunol 30:607-617

Masek T, Vopalensky V, Suchomelova P, Pospisek M (2005) Denaturing RNA electrophoresis in TAE agarose gels. Anal Biochem 336:46-50

Matsubara H, Nakamura-Tsuruta S, Hirabayashi J, Jimbo M, Kamiya H, Ogawa T, Muramoto K (2007) Diverse sugar-binding specificities of marine invertebrate C-type lectins. Biosci Biotechnol Biochem 71:513-519

Multhoff G (2006) Heat shock proteins in immunity. Handb Exp Pharmacol 172:279-304

O'Leary NA, Gross PS (2006) Genomic structure and transcriptional regulation of the penaeidin gene family from *Litopenaeus vannamei*. Gene 371:75-83

Roberts RJ, Agius C, Saliba C, Bossier P, Sung YY (2010) Heat shock proteins (chaperones) in fish and shellfish and their potential role in relation to fish health: a review. J Fish Diseases 33:789-801

Schnapp D, Kemp GD, Smith VJ (1996) Purification and characterization of a proline-rich antibacterial peptide, with sequence similarity to bactenecin-7, from the haemocytes of the shore crab, *Carcinus maenas*. Eur J Biochem 240:532-539

Smith VJ, Fernandes JM, Kemp GD, Hauton C (2008) Crustins: enigmatic WAP domain-containing antibacterial proteins from crustaceans. Dev Comp Immunol 32:758-772

Tan Y, Xing Y, Zhang H, Feng Y, Zhou Y, Shi ZL (2009) Molecular detection of three shrimp viruses and genetic variation of white spot syndrome virus in Hainan Province, China, in 2007. J Fish Diseases 32:777–784

Tang KF, Lightner DV (2006) Infectious hypodermal and hematopoietic necrosis virus (IHHNV)-related sequences in the genome of the black tiger prawn *Penaeus monodon* from Africa and Australia. Virus Res 118:185-191

Tassanakajon A, Amparyup P, Somboonwiwat K, Supungul P (2011) Cationic antimicrobial peptides in penaeid shrimp. Mar Biotechnol 13:639-657

Teixeira MA, Cruz JEF, Vieira PRN, Branco IRC, Costa FHF, Rádis-Baptista G (2010) Differential diagnosis of active hypodermal and hematopoietic necrosis virus based on gene choice and reverse transcription coupled with PCR. Genet Mol Res 9:2025-2031

Teixeira-Lopes MA, Vieira-Girão PRN, Freire JEC, Rocha IRCB, Costa FHF, Rádis-Baptista G (2011) Natural co-infection with infectious hypodermal and hematopoietic necrosis virus (IHHNV) and infectious myonecrosis virus (IMNV) in *Litopenaeus vannamei* in Brazil. Aquaculture 312:212-216

Tsan MF, Gao B (2004) Cytokine function of heat shock proteins. Am J Physiol Cell Physiol 286:739-744

Vatanavicharn T, Supungul P, Puanglarp N, Yingvilasprasert W, Tassanakajon A (2009) Genomic structure, expression pattern and functional characterization of crustinPm5, a unique isoform of crustin from *Penaeus monodon*. Comp Biochem Physiol B Biochem Mol Biol 153:244-252

Wang PH, Wan DH, Gu ZH, Deng XX, Weng SP, Yu XQ, He JG (2011) *Litopenaeus vannamei* tumor necrosis factor receptor-associated factor 6 (TRAF6) responds to *Vibrio alginolyticus* and white spot syndrome virus (WSSV) infection and activates antimicrobial peptide genes. Dev Comp Immunol 35:105-114

Wood-Charlson EM, Weis VM (2009) The diversity of C-type lectins in the genome of a basal metazoan, *Nematostella vectensis*. Dev Comp Immunol 33:881-889

Wu JL, Nishioka T, Mori K, Nishizawa T, Muroga K (2002) A time-course study on the resistance of Penaeus japonicus induced by artificial infection with white spot syndrome virus. Fish & Shellfish Immunol 13:391–403

Yamaura K, Takahashi KG, Suzuki T (2008) Identification and tissue expression analysis of C-type lectin and galectin in the Pacific oyster, *Crassostrea gigas*. Comp Biochem Physiol B Biochem Mol Biol 149:168-175

Zhang H, Wang H, Wang L, Song X, Zhao J, Qiu L, Li L, Cong M, Song L (2009) A novel C-type lectin (Cflec-3) from *Chlamys farreri* with three carbohydrate-recognition domains. Fish Shellfish Immunol 26:707-715

Zhao ZY, Yin ZX, Xu XP, Weng SP, Rao XY, Dai ZX, Luo YW, Yang G, Li ZS, Guan HJ, Li SD, Chan SM, Yu XQ, He JG (2009) A novel C-type lectin from the shrimp *Litopenaeus vannamei* possesses anti-white spot syndrome virus activity. J Virol 83:347-56

Zhou J, Wang WN, He WY, Zheng Y, Wang L, Xin Y, Liu Y, Wang AL (2010) Expression of HSP60 and HSP70 in white shrimp, *Litopenaeus vannamei* in response to bacterial challenge. J Invertebr Pathol 103:170-178

Table 1 Primer sequences for quantitative real-time PCR analysis of shrimp viruses and host innate immunity-related genes

L. vannamei mRNA and virus genes	GenBank accession number	Forward (F) and reverse (R) primers	Sequence (5' → 3')	Amplicon size (nt)
IHHNV	gb|AF218266|	IV77012F	TTATGTGCATCCCTCCTGGAT	356
		IV77353R	TCGTACTGGCTGTTCATCCT	
IMNV	gb|AY570982|	MV4587F	CGACGCTGCTAACCATACAA	328
		MV4914R	ACTCGGCTGTTCGATCAAGT	
β-actin	gb|AF300705|	bAct F02	CCACGAGACCACCTACAAC'	183
		bAct R01	TTAGAAGCACTTCCTGTGAACAAT	
Crustin	gb|AF430076|	CTN F01	CACGAGGCAACCATGAAGG	142
		CTN R02	TCTTGCACCAATACCTGCAGT	
C-type lectin (CTL-br1)	gb|GU206551|	CTLbr1 GTR (F)	ATCCAGGAACCCGATGGAGGA	102
		CTLbr1 CRD2 (R)	TTATCCAGTATAGACACACAGTGGAT	
HSP-70	gb|AY645906|	HSP-70 F01	ATGGCAAAGGCACCTGCTGT	373
		HSP-70 R02	TGAGCACCATCGAGGAGATCT	
Penaeidin-3a	emb|Y14926|	Pen3 F02	AGAGACCGACGCTCCGAG	141
		Pen3 R01	AATATCCCTTTCCCACGTGAC	

Table 2 Classification of farmed shrimp samples based on gross signal of viral disease

Group	Number (nr) of shrimp samples and corresponding percentage (%)	IHHNV (+)	IMNV (+)	Co-infection[a] (IHHNV + IMNV)
Asymptomatic	50 (100%)	98%	nd[b]	nd[b]
Showing gross signal of				
IHHNV	40 (100%)	20 (50%)	05 (12.5%)	15 (37.5%)
IMNV	30 (100%)	03 (10%)	02 (6.7%)	22 (75%)

[a]Samples diagnosed by RT-PCR-based technique.
[b] IMNV presence was not determined (nd) in shrimp samples initially diagnosed for IHHNV.

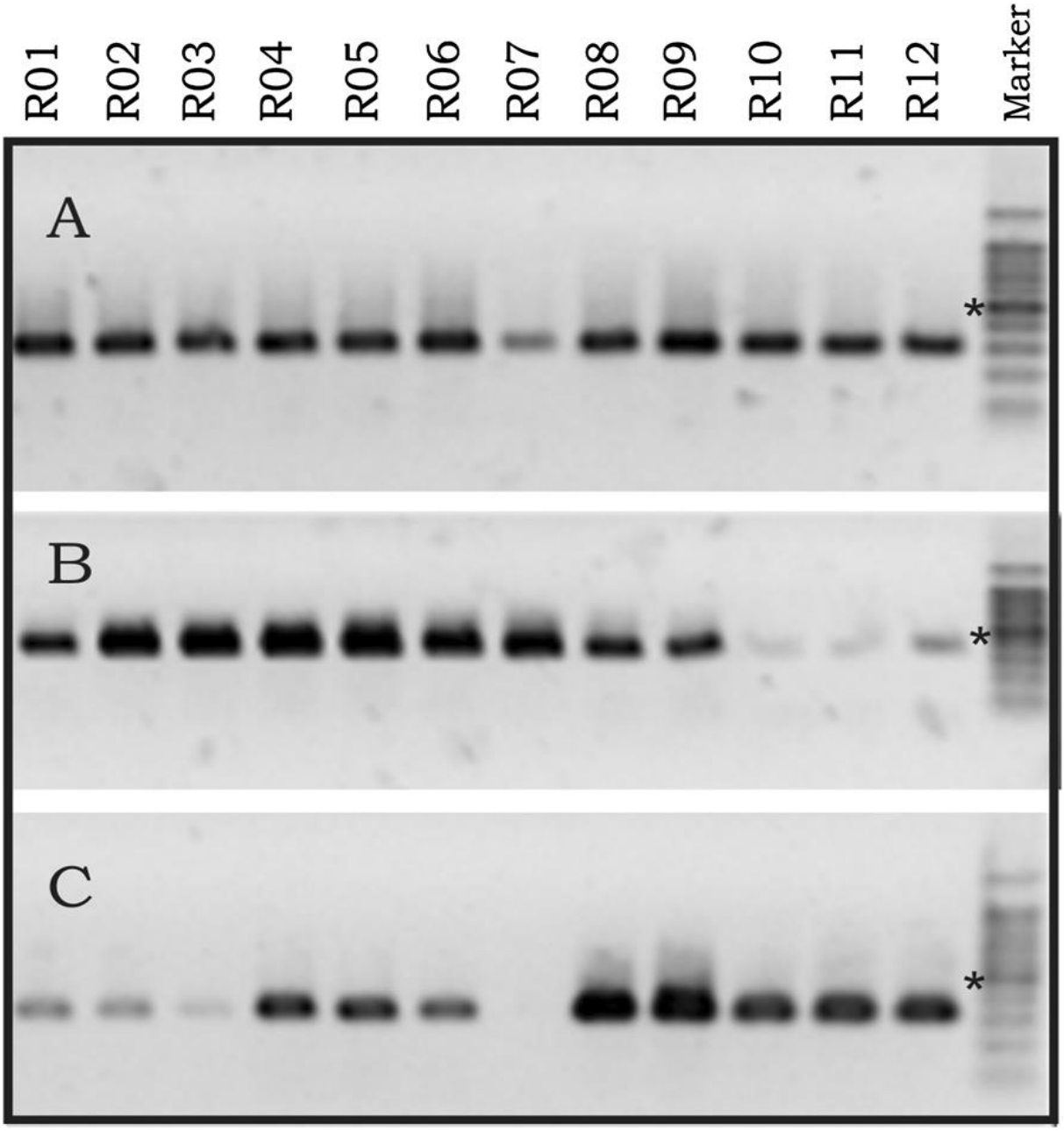
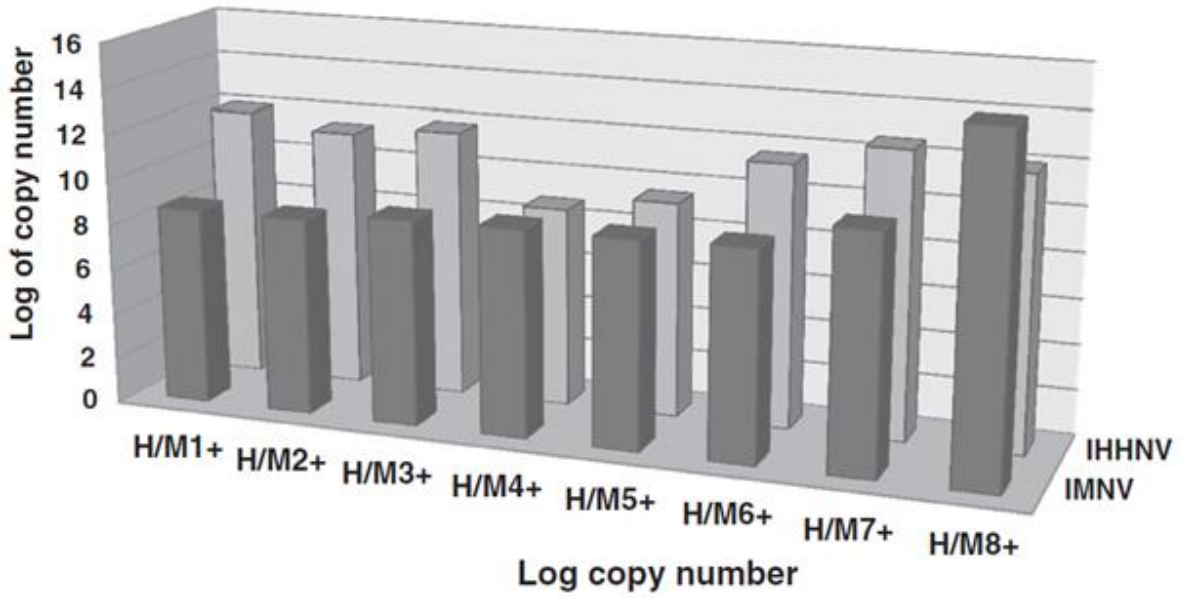


Figure 1. Qualitative diagnostic analysis of IHHNV and IMNV by reverse transcription coupled with PCR (RT-PCR). (A) The product of the amplification reaction of the penaeid β-actin gene that was used as internal control. (B) Representative samples that are positive for IHHNV, amplified from cDNA using the primer pair for IHHNV detection as described in Table 1. In (C), the same samples that are shown in (A) and (B); however, the samples were analyzed for IMNV infection using the primers MV4587F and MV4914R (Table 1). The lanes in each individual position, in all panels, correspond to the same sample. Marker = 1 bp DNA ladder. Asterisks denote 500 bp.



	H/M1+	H/M2+	H/M3+	H/M4+	H/M5+	H/M6+	H/M7+	H/M8+
IMNV	8.66	8.67	9.06	9.12	9.12	9.23	10.4	14.92
IHHNV	12	11.4	11.8	8.8	9.5	11.6	12.6	12

Figure 2 Reciprocal quantitative differences in shrimp samples co-infected with IHHNV and IMNV. The logarithm of the mean copy number of IHHNV and IMNV co-infecting farmed *L. vannamei*. In each shrimp sample ($n \geq 6$), actively transcribed genes from IHHNV and IMNV were quantified by absolute qPCR using cDNA as the template and specific primers as described in Table 1. The number of viral particles was then normalized in relation to the logarithm (Log_{10}) of copy number of 1 μg of shrimp β actin transcripts.

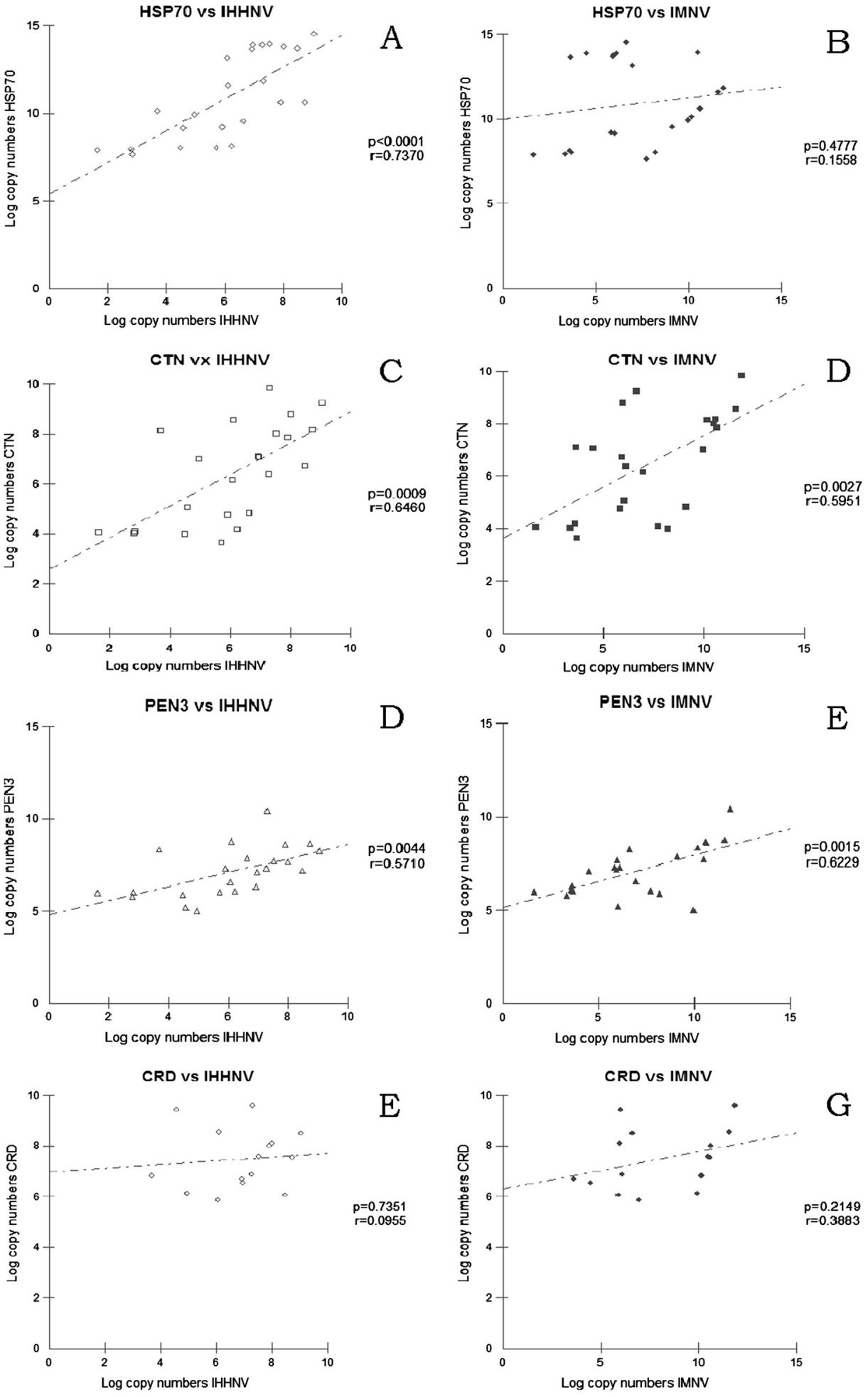

HSP70 vs IHHNV
A
p<0.0001
r=0.7370
Log copy numbers HSP70
Log copy numbers IHHNV
HSP70 vs IMNV
B
p=0.4777
r=0.1558
Log copy numbers HSP70
Log copy numbers IMNV
CTN vx IHHNV
C
p=0.0009
r=0.6460
Log copy numbers CTN
Log copy numbers IHHNV
CTN vs IMNV
D
p=0.0027
r=0.5951
Log copy numbers CTN
Log copy numbers IMNV
PEN3 vs IHHNV
D
p=0.0044
r=0.5710
Log copy numbers PEN3
Log copy numbers IHHNV
PEN3 vs IMNV
E
p=0.0015
r=0.6229
Log copy numbers PEN3
Log copy numbers IMNV
CRD vs IHHNV
E
p=0.7351
r=0.0955
Log copy numbers CRD
Log copy numbers IHHNV
CRD vs IMNV
G
p=0.2149
r=0.3883
Log copy numbers CRD
Log copy numbers IMNV

Figure 3 Linear correlations between shrimp virus copy number and selected genes from the innate immune system. (A) IHHNV vs. HSP-70; (B) IMNV vs. HSP-70; (C) IHHNV vs. crustin; (D) IMNV vs. crustin; (E) IHHNV vs. penaeidin-3a; (F) IMNV vs. penaeidin-3a; (G) IHHNV vs. C-type lectin (CTL)-br1; (H) IMNV vs. CTL-br1. Quantitative (q) PCR data were obtained and statistically analyzed as described in the 'Methods' section. The values of r and P are shown in the plots.

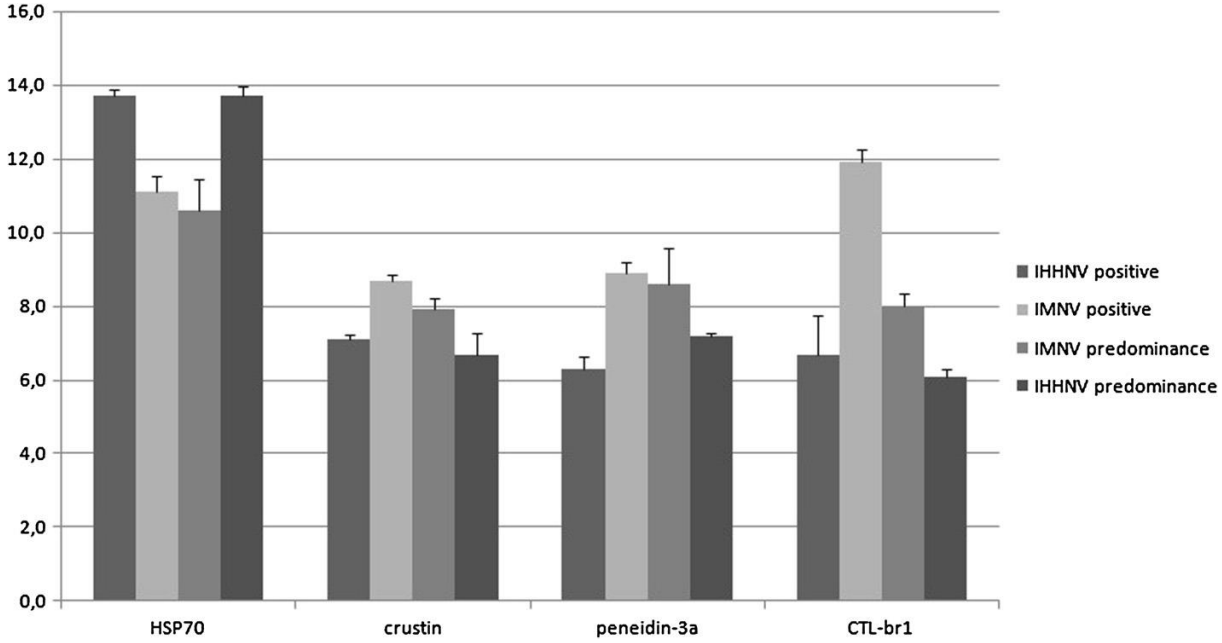


Figure 4 Comparative expression: innate immune genes of shrimp infected with a virus alone or simultaneously co-infected. Representative shrimp samples ($n \geq 9$) that are positive only for IHHNV or IMNV, as well as positive for both viruses were confirmed by RT-PCR, and the viral load (log of copy number) was determined by absolute qPCR. The samples labeled 'Positive' are those in which the log copy number of viral particle is maximal for one type of virus and 'zero' for the another; 'Predominance' means that a kind of virus that predominates (in terms of copy number) in relation to the other for the same numerical factor; for example, in the sample labeled as 'IHHNV predominance' the difference between IHHNV and IMNV is 2.6, while in IMNV predominance, the difference between IMNV and IHHNV is 2.7. $P < 0.01$.

8 CONCLUSÕES GERAIS

- Os vírus, IHHNV e IMNV, ocorrem simultaneamente em brânquias de camarões infectados em fazendas do Nordeste do Brasil;

- A expressão de HSP-70 foi aumentada em resposta ao aumento da replicação de IHHNV;

- O disparo no aumento da expressão de HSP-70 parece ser um fator que facilita a entrada e propagação do IMNV, que é, na maioria das vezes, o responsável pela morte dos camarões;

- A crustina apresentou expressão aumentada em resposta a replicação dos vírus, porém com maior correlação também com o IHHNV;

- A peneidina-3ª apresentou expressão também aumentada em resposta a replicação dos vírus, no entanto observou-se maior correlação com o IMNV;

- A expressão de CTL-br1 não apresentou correlação com a replicação de nenhum do vírus;

- Através do estudo de detecção e quantificação dos vírus que infectavam os camarões coletados naquele momento, pôde-se observar que em algum instante um vírus prevalece sobre o outro, embora os camarões estivessem portando os dois vírus. Nesse contexto, a técnica de RT-PCR utilizada nesse estudo, pode ser empregada em diagnósticos de rotina, e PCR em tempo real pode ser uma ferramenta diferencial na prevenção de surtos de doenças pela detecção de pequenas quantidades de vírus, antes de aparecerem os sintomas, em estágios mais iniciais da infecção.

9 PERSPECTIVAS

- A partir do estudo sobre a correlação de moléculas da imunidade de camarões infectado pelos vírus, IHHNV e IMNV, observou-se que a HSP-70 constitui uma molécula passível de utilização como biomarcador molecular no monitoramento do estado de saúde de camarões cultivados, bem como na seleção genética de animais com melhor resposta imunológica e mais resistentes a infecção viral para compor um plantel de reprodutores;

- Mais estudos que analisem mudanças moleculares nos estágios mais iniciais da infecção, com testes *in vivo* em laboratório e análises com PCR em tempo real, têm sido realizados pelo nosso grupo para a um melhor entendimento das respostas imunes em *L. vannamei*;

- Diante dos resultados preliminares obtidos com os testes *in vivo* e visando encontrar soluções para os problemas de enfermidades no cultivo (em campo e laboratório) de *L. vannamei*, um estudo com o desenvolvimento e manutenção de linhagens de células derivadas de diferentes tecidos de *L. vannamei*, bem como o estudo da expressão e da regulação de genes da imunidade inata, apoptose e necrose, durante as fases iniciais e tardias da infecção e replicação viral, é o objetivo de um projeto de doutoramento já aprovado pela Rede Nordeste de Biotecnologia (RENORBIO), com início em 2013.

REFERÊNCIAS

ASSOCIAÇÃO BRASILEIRA DE CRIADORES DE CAMARÃO - ABCC (2011). *Carcinicultura Brasileira: Processos Tecnológicos, Impactos Sócio-Econômicos, Sustentabilidade Ambiental, Entraves e Oportunidades*. Diretor da ABCC, Itamar de Paiva Rocha. Site: www.abbccam.com.br, acesso: nov/2012.

BACHÈRE, E. (2000). Shrimp immunity and disease control. *Aquaculture* 191: 3-11.

BARBIERI, J. R. C.; OSTRESKY, N. A. *Camarões marinhos: Engorda*. Viçosa: Aprenda fácil, 2002.

BARRACCO, M. A. *Mecanismos de resistência a doenças em crustáceos*, *In*: Sanidade de organismos aquáticos, S. I.: Varela, p. 51-74, 2004.

BONAMI, J. R.; BREHELIN, M.; MARI, J.; TRUMPER, B.; LIGHTNER, D. V. Purification and characterization of IHHN virus of penaeid shrimps. *J Gen Virol*, v. 71, p. 2657-2664, 1990.

BRASIL (2007). Diretoria de Fauna e Recursos Pesqueiros. Estatística da Pesca, 2005: Grandes Regiões e Unidades da Federação. IBAMA (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis), Brasília.

BUSTIN, S. A.; BENES, V.; GARSON, J. A.; HELLEMANS, J.; HUGGETT, J.; KUBISTA, M.; MUELLER, R.; NOLAN, T.; PFAFFL, M. W.; SHIPLEY, G. L.; VANDESOMPELE, J.; WITTWER, C. T. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR. *Exper Clin Chem*, v. 55 (4), p. 611–622, 2009.

CLAVERO-SALAS, A.; SOTELO-MUNDO, R. R.; GOLLAS-GALVAN, T.; HERNÁNDEZ-LÓPEZ, J.; PEREGRINO-URIARTE, A. B.; MUHLIA-ALMAZAN, A.; YEPIZ-PLASCENCIA, G. Transcriptome analysis of gills from the white shrimp *Litopenaeus vannamei* infected with White Spot Syndrome Virus. Fish Shellfish Immunol, v. 23, p. 459-472, 2007.

COSTA, F. H. F.; VALENÇA, N. S.; SILVA, A. R.; BEZERRA, G. A.; CAVADA, B. S.; RÁDIS-BAPTISTA, G. Cloning and molecular modeling of *Litopenaeus vannamei* (Penaeidae) C-type lectin homologs with mutated mannose binding domain-2. *Genet Mol Res*, v. 10, p. 650–664, 2011.

DESTOUMIEUX, D.; BULET, P.; LOEW, D.; VAN DORSSELAER, A.; RODRIGUEZ, J.; BACHÈRE, E. Penaeidins, a new family of antimicrobial peptides isolated from the shrimp *Penaeus vannamei* (Decapoda). *J Biol Chem*, v. 272, p. 28398–28406, 1997.

FAO (2012). Organización de las Naciones Unidas para la Alimentación y la Agricultura. EL ESTADO MUNDIAL DE LA PESCA Y LA ACUICULTURA. Departamento de Pesca y Acuicultura de la FAO, Roma.

FUJITA, T.; MATSUSHITA, M.; ENDO, Y. The lectin-complement pathway–its role in innate immunity and evolution. *Immunol Rev*, v. 198, p. 185–202, 2004.

GROSS, P. S.; BARTLETT, T. C.; BROWDY, C. L.; CHAPMAN, R. W.; WARR, G. W. Immune gene discovery by expressed sequence tag analysis of hemocytes and hepatopancreas in the Pacific white shrimp, *Litopenaeus vannamei*, and the Atlantic white shrimp, *L. setiferus*. *Dev Comp Immunol*, v. 25, p. 565–577, 2001.

IWANAGA, S.; LEE, B. L. Recent advances in the innate immunity of invertebrate animals. *J Bioch Mol Biol*, v. 38, p. 128–150, 2005.

HICKMAN, C. P.; ROBERTS, L. S.; LARSON, A. *Princípios Integrados de Zoologia*. Rio de Janeiro: Guanabara Koogan S.A, 2004.

HUANG, W. J.; LEU, J. H.; TSAU, M. T.; CHEN, J. C.; CHEN, L. L. Differential expression of LvHSP60 in shrimp in response to environmental stress. *Fish Shellfish Immunol*, v. 30, p. 576–582, 2011.

JIAO, C. Z.; WANG, Z. Z.; LI, F. H.; ZHANG, C. S.; XIANG, J. H. Cloning, sequencing and expression analysis of cDNA encoding a constitutive heat shock protein 70 (HSC70) in *Fenneropenaeus chinensis*. *Chin Sci Bull*, v. 49, p. 2385–2393, 2004.

JOVENTINO, F K P; MAYORGA, M I O (2008). Diagnóstico socioambiental e tecnológico da carcinicultura no município de Fortim, Ceará, Brasil. *Rev Eletr Prodema,* 2: 80-96.

KALAGAYAN, G; GODIN, D; KANNA, R; HAGINO, G; SWEENEY, J; WYBAN, J; BROCK, J (1991). IHHN virus as an etiological factor in runt-deformity syndrome of juvenile Penaeus vannamei cultured in Hawaii. *J World Aquac Soc*, 22: 235–243.

KAMIMURA, M T; MEIER, K M; CAVALLI, R O; LAURINO, J; MAGGIONI, R; MARINS, L F (2008). Characterization of growth-related genes in the south-western Atlantic Pink shrimp *Farfantepenaeus paulensis* (Pérez-Farfante, 1967) throung a modifield DDRT-PCR protocol. *Aquac Res*, 39: 200-204.

KONG, H J; PARK, E M; NAM, B H; KIM, Y O; KIM, W J; PARK, H J; LEE, C H; LEE, S J (2008). A C type lectin like-domain (CTLD)-containing protein (PtLP) from the swimming crab Portunus trituberculatus. *Fish Shellfish Immunol*, 25: 311–314.

LI, C; WENG, S; CHEN, Y; YU, X; LU, L; ZHANG, H; HE, J; XU, X (2012). Analysis of *Litopenaeus vannamei* Transcriptome Using the Next-Generation DNA Sequencing Technique. *PLoS ONE*, 7(10): 47442.

LIGHTNER, D V; REDMAN, R M; BELL, T A (1983). Infectious hypodermal and hematopoietic necrosis, a newly recognized virus disease of penaeid shrimp. *J Invertebr Pathol*, 42: 62–70.

LIGHTNER, D V; PANTOJA, C R; POULOS, B T; TANG, K F J; REDMAN, R M; PASOS DE ANDRADE, T; BONAMI, J R (2004). Infectious myonecrosis: new disease in Pacific white shrimp. *Glob Aquac Advocate*, 7: 85.

LIGHTNER, D V (2011). Virus diseases of farmed shrimp in the Western Hemisphere (the Americas): A review. *J Invert Pathol*, 106: 110-130.

LIGHTNER, D V (2012). Historic emergence, impact and current status of shrimp pathogens in the Americas. *J Invert Pathol*, 110: 174–183.

LIU, Y C; LI, F H; DONG, B; WANG, B; LUAN, W; ZHANG, X J; ZHANG, L S; XIANG J H (2007). Molecular cloning, characterization and expression analysis of a putative C-type lectin (Fclectin) gene in Chinese shrimp *Fenneropenaeus chinensis*. *Mol Immunol*, 44: 598–607.

LUO, T; YANG, H; LI, F; ZHANG, X; XU, X (2006). Purification, characterization and cDNA cloning of a novel lipopolysaccharide-binding lectin from the shrimp *Penaeus monodon*. *Dev Comp Immunol*, 30: 607–617.

MARI, J; BONAMI, J R; LIGHTNER, D V (1993). Partial cloning of the genome of infectious hypodermal and haematopoietic necrosis virus, an unusual parvovirus pathogenic for penaeid shrimps; diagnosis of the disease using a specific probe. *J Gen Virol*, 74: 2637–2643.

MATSUBARA, H; NAKAMURA-TSURUTA, S; HIRABAYASHI, J; JIMBO, M; KAMIYA, H; OGAWA, T; MURAMOTO, K (2007). Diverse sugar-binding specificities of marine invertebrate C-type lectins. *Biosci Biotechnol Biochem*, 71: 513–519.

MOTTE, E; YUGCHA, E; LUZARDO, J CASTRO, F; LECLERCQ, G; RODRGUEZ, J; MIRANDA, P; BORJA, O; SERRANO, J; TERREROS, M; MONTALVO, K; NARVAEZ, A; TENORIO, N; CEDENO, V; MIALHE, E; BOULO, V (2003). Pevention of IHHNV vertical transmission in the white shrimp *Litopenaeus vannamei*. *Aquaculture*, 219: 57-70.

MUÑOZ, M; VANDENBULCKE, F; SAULNIER, D; BACHÈRE, E (2002). Expression and distribution of penaeidin antimicrobial peptides are regulated by haemocyte reactions in microbial challenged shrimp. *Eur J Biochem*, 269: 2678–2689.

MUÑOZ, M; VANDENBULCKE, F; GUEGUEN, Y (2003). Expression of penaeidin antimicrobial peptides in early larval stages of the shrimp *Penaeus vannamei*. *Devel Comp Immunol*, 27: 283–289.

MUÑOZ, M; VANDENBULCKE, F; GARNIERA, J; GUEGUEN, Y; BULET, P; SAULNIER, D; BACHÈRE, E (2004). Involvement of penaeidins in defense reactions of the shrimp *Litopenaeus stylirostris* to a pathogenic vibrio. *Cell Mol Life Sci*, 61: 961–972.

OIE (2009). Manual of Diagnostic Tests for Aquatic Animal Diseases. Office International des Epizooties (OIE), Paris, France. Ed. 6, 96-104p.

O'LEARY, N A; GROSS, P S (2006). Genomic structure and transcriptional regulation of the penaeidin gene family from *Litopenaeus vannamei*. *Gene*, 371: 75–83.

PAN, D; HE, N; YANG, Z; LIU, H; XU, X (2005). Differential gene expression profile in hepatopancreas of WSSV-resistant shrimp (*Penaeus japonicus*) by suppression in WSSV-infected *Penaeus monodon* Fabricius. *Aquaculture*, 254: 32–39.

PFAFFL, M W (2001). A new mathematical model of relative quantification in real-time RT-PCR. *Nucleic Acid Res*, 29 (9): 2003-2007.

POULOS, B T; LIGHTNER, D V (2006). Detection of infectious myonecrosis virus (IMNV) of penaeid shrimp by reverse-transcriptase polymerase chain reaction (RT-PCR). *Dis Aquat Organ*, 73: 69–72.

POULOS, B T; TANG, K F J; PANTOJA, C R; BONAMI, J R; LIGHTNER, D V (2006). Purification and characterization of infectious myonecrosis virus of penaeid shrimp. *J Gen Virol*, 87: 987–996.

QIAN, Z; LIU, X; WANG, L; WANG, X; LI, Y; XIANG, J; WANG, P (2012). Gene expression profiles of four heat shock proteins in response to different acute stresses in shrimp, *Litopenaeus vannamei*. *Comp Biochem Physiol C*, 156: 211–220.

ROBERTS, R J; AGIUS, C; SALIBA, C; BOSSIER, P; SUNG, Y Y (2010). Heat shock proteins (chaperones) in fish and shellfish and their potential role in relation to fish health: a review. *J Fish Diseases*, 33: 789–801.

ROSA, G J M; ROCHA, L B; FURLAN, L R (2007). Estudos de expressão gênica utilizando-se *microarrays*: delineamento, análise, e aplicações na pesquisa zootécnica. *R Bras Zootec*, 36: 185-209.

RUNGRASSAMEE, W; LEELATANAWIT, R; JIRAVANICHPAISAL, P; KLINBUNGA, S; KAROONUTHAISIRI, N (2010). Expression and distribution of three heat shock protein genes under heat shock stress and under exposure to *Vibrio harveyi* in *Penaeus monodon*. *Dev Comp Immunol*, 34: 1082–1089.

SCHNAPP, D; KEMP, G D; SMITH, V J (1996). Purification and characterization of a proline rich antibacterial peptide, with sequence similarity to bactenecin-7, from the haemocytes of the shore crab, *Carcinus maenas*. *Eur J Biochem*, 240: 532–539.

SENAPIN, S; PHEWSAIYA, K; BRIGGS, M; FLEGEL, T W (2007). Outbreaks of infectious myonecrosis virus (IMNV) in Indonesia confirmed by genome sequencing and use of an alternative RT-PCR detection method. *Aquaculture*, 22: 32–38.

SIMPSON, D A C; FEENEY, S; BOYLE, C; STITT, A W (2000). Retinal VEGF mRNA measured by SYBR Green I fluorescence: versatile approach to quantitative PCR. *Molecular Vision*, 6: 178-83.

SMITH, V J; FERNANDES, J M; KEMP, G D; HAUTON, C (2008). Crustins: enigmatic WAP domain-containing antibacterial proteins from crustaceans. *Dev Comp Immunol*, 32: 758–772.

TAN, Y; XING, Y; ZHANG, H; FENG, Y; ZHOU, Y; SHI, Z L; (2009). Molecular detection of three shrimp viruses and genetic variation of white spot syndrome virus in Hainan Province, China, in 2007. *J Fish Dis*, 32: 777–784.

TANG, K F; LIGHTNER, D V (2006). Infectious hypodermal and hematopoietic necrosis virus (IHHNV)-related sequences in the genome of the black tiger prawn *Penaeus monodon* from Africa and Australia. *Virus Res*, 118: 185–191.

TANG, J; OCHOA, W F; SINKOVITS, R S; POULOS, B T; GHABRIAL, S A; LIGHTNER, D V; BAKER, T S; NIBERT, M L (2008). Infectious myonecrosis virus has a totivirus-like, 120-subunit capsid, butwithfiber complexes at thefivefoldaxes. *Proc Natl Acad Sci USA*, 105: 17526–17531.

TEIXEIRA, M A; CRUZ, J E F; VIEIRA, P R N; BRANCO, I R; COSTA, F H F; RÁDIS-BAPTISTA, G (2010). Differential diagnosis of active hypodermal and hematopoietic necrosis virus based on gene choice and reverse transcription coupled with PCR. *Genet Mol Res*, 9: 2025–2031.

VAN DE BRAAK, C B; BOTTERBLOM, M H A; TAVERNE, N; VAN MUISWINKEL, W B; ROMBOUT, J H W M (2002). The role of the haematopoietic tissue in haemocyte production and maturation in the black tiger shrimp (*Penaeus monodon*). *Fish Shellfish Immun*, 12: 253-272.

VANPATTEN, K A; NUNAN, L M; LIGHTNER, D V (2004). Seabirds as potential vectors of penaeid shrimp viruses and the development of a surrogate laboratory model utilizing domestic chickens. *Aquaculture*, 241: 31-46.

VATANAVICHARN, T; SUPUNGUL, P; PUANGLARP, N; YINGVILASPRASERT, W; TASSANAKAJON, A (2009). Genomic structure, expression pattern and functional characterization of crustinPm5, a unique isoform of crustin from *Penaeus monodon*. *Comp Biochem Physiol B Biochem Mol Biol*, 153: 244–252.

VEGA, E; HALL, M R; DEGNAN, B M; WILSON, K J (2006). Short-term hyperthermic treatment of *Penaeus monodon* increases expression of heat shock protein 70 (HSP70) and reduces replication of gill associated virus (GAV). Aquaculture, 253: 82– 90.

WANG, W; ZHANG, X (2008). Comparison of antiviral efficiency of immune responses in shrimp. *Fish Shellfish Immun*, 25: 522-527.

WONG, M L; MEDRANO J F (2005). Real-time PCR for mRNA quantitation. *BioTechniques*, 39 (1): 1-11.

WOOD-CHARLSON, E M; WEIS, V M (2009). The diversity of C-type lectins in the genome of a basal metazoan, *Nematostella vectensis*. *Dev Comp Immunol*, 33: 881–889.

WU, R; SUN, Y; LEI, L; XIE, S (2008). Molecular identification and expression of heat shock cognate 70 (HSC 70) in the pacific white shrimp *Litopenaeus vannamei*. *Mol. Biol*, 42: 234–242.

YAMAURA, K; TAKAHASHI, K G; SUZUKI, T (2008). Identification and tissue expression analysis of C-type lectin and galectin in the Pacific oyster, *Crassostrea gigas*. *Comp Biochem Physiol B Biochem Mol Biol*, 149: 168–175.

YAN, D C; TANG, K F J; LIGHTNER, D V (2010). A real-time PCR for the detection of hepatopancreatic parvovirus (HPV) of penaeid shrimp. *J Fish Dis*, 33: 507–511.

YU, X W; WANG, J P; ZHANG, W; SHI, Z L (2011). Prevalence of Three Shrimp Viruses in Zhejiang Province in 2008. *Vir Sin*, 26: 67-71.

YUE, Z Q; LIU, H; WANG, W; LEI, Z W; LIANG, C Z; JIANG, Y L (2006). Development of real time polymerase chain reaction assay with TaqMan probe for the quantitative detection of infectious hypodermal and hematopoietic necrosis virus from shrimp. *J AOAC Int*, 89: 240–244.

ZHANG, H; WANG, H; WANG, L; SONG, X; ZHAO, J; QIU, L; LI, L; CONG, M; SONG, L (2009). A novel C-type lectin (Cflec-3) from *Chlamys farreri* with three carbohydrate-recognition domains. *Fish Shellfish Immunol*, 26: 707–715.

ZHOU, J; WANG, W N; MA, G Z; WANG, A L; HE, W Y; WANG, P; LIU, Y; LIU, J J; SUN, R Y (2008). Gene expression of ferritin in tissue of the Pacific white shrimp, *Litopenaeus vannamei* after exposure to pH stress. *Aquaculture*, 275: 356–360.

ZHOU, J; WANG, L; XIN, Y; WANG, W N; HE, W Y; WANG, A L; LIU, Y (2010a). Effect of temperature on antioxidant enzyme gene expression and stress protein response in white shrimp, *Litopenaeus vannamei*. *J Therm Biol*, 35: 284–289.

ZHOU, J; WANG, W N; HE, WY ; ZHENG, Y; WANG, L; XIN, Y; LIU, Y; WANG, A L (2010b). Expression of HSP60 and HSP70 in white shrimp, *Litopenaeus vannamei* in response to bacterial challenge. *J Invert Pathol*, 103: 170–178.

ZIPPER, H; BRUNNER, H; BERNHAGEN, J; VITZTHUM, F (2004). Investigations on DNA intercalation and surface binding by SYBR Green I, its structure determination and methodological implications. *Nucleic Acids Research*, 32 (12): 103.

APÊNDICES