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DEVELOPMENT OF RT-qPCR METHODOLOGIES FOR THE DETECTION OF VIRAL PATHOGENS IN VARIOUS SHRIMP SPECIES.

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ABSTRACT

Arseneau, J.R. and Laflamme, M. 2016. Development of RT-qPCR methodologies for the detection of viral pathogens in crustaceans of commercial importance. Can. Tech. Rep. Fish. Aquat. Sci. 3134: iii+21pp.

Epizootics in crustaceans are a concern for any country relying on aquaculture trade as a major source of revenue. Prevention of introductions and outbreaks of shrimp pathogens relies on robust testing methods. The recent influx of genomic data for many pathogens is allowing the development of better tools for the early detection of these pathogens before they can cause outbreaks. We have chosen five pathogens of international importance according to the ‘‘Office International des Epizooties’’ that are considered immediately notifiable according to Canadian law (http://www.gazette.gc.ca/rp-pr/p2/2011/2011-01-05/html/sor-dors310-eng.html). These pathogens are IHHNV, MrNV, IMNV, YHV and TSV. We have developed very sensitive and quantitative nucleic acid-based assays for each of these pathogens. Each assay was analytically bench-validated by evaluating their specificity and calculating the limits of detection. Further, these assays were developed as a suite of assays, using common reagents and equipment settings whenever possible. We suggest that these assays are most suited for surveillance purposes.

Keywords : RT-qPCR, validation, test, shrimp, virus, pathogen, IHHNV, WTD, IMNV, YHV, TSV

RÉSUMÉ

Arseneau, J.R. et Laflamme, M. 2016**.** Development of RT-qPCR methodologies for the detection of viral pathogens in crustaceans of commercial importance. Rapp. Tech. Can. Sci. Halieut. et Aquat. 3134: iii+21pp.

L’apparition d’épizootiques chez les crustacés est une préoccupation pour tous les pays appuyant le commerce de l'aquaculture comme une source importante de revenus. La prévention d’introductions ou d’éclosions de pathogène repose sur le développement tests robustes. L'afflux récent de données génomiques pour de nombreux agents pathogènes permet le développement de meilleurs outils pour la détection précoce de ces agents pathogènes avant qu'ils ne puissent causer des épidémies. Nous avons choisi cinq agents pathogènes d'importance internationale selon le " Office international des épizooties " qui sont considérés à notification immédiate conformément à la loi canadienne (http://www.gazette.gc.ca/rp-pr/p2/2011/2011-01-05/html/sor-dors310-eng.html). Ces pathogènes sont IHHNV, MrNV, IMNV, YHV et TSV. Nous avons développé des tests à base d'acides nucléiques très sensibles et quantitatifs pour chacun de ces agents pathogènes. Chaque essai a été validé analytiquement par l'évaluation de leur spécificité et avec le calcul des limites de détection. En outre, ces tests ont été développés comme un ensemble intègre, utilisant des réactifs et des réglages d'appareil communs tant que possible. Nous suggérons que ces tests sont les plus adaptés à des fins de surveillance.

Mots-clés : RT-qPCR, validation, test, crevette, virus, pathogène, IHHNV, WTD, IMNV, YHV, TSV

INTRODUCTION

Shrimp aquaculture has long been a major source of revenue for many countries spread out all over the globe. For example, in 2009, about 3.5 million tons of shrimp and prawn were produced worldwide, generating revenue of over 14 billion US dollars (ftp://ftp.fao.org/FI/CDrom/CD_yearbook_2009/root/aquaculture/b1.pdf). Despite this success, the industry has had to deal with the looming threat of infectious diseases that lead to mass mortalities and massive losses in revenue (Dhar et al., 2014; Leung et al., 2000; Lightner et al., 2012; Moss et al., 2012). As of 2015, the World Organisation for Animal Health, or ''Office International des Epizooties'' (OIE), has listed eight different crustacean diseases that are immediately notifiable (http://www.oie.int/animal-health-in-the-world/oie-listed-diseases-2015/). Although some of these diseases are found only in specific parts of the world, others have world-wide distributions (Lightner et al., 2012). Although shrimp aquaculture is in its infancy in Canada, there is a thriving wild fishery. In 2014, shrimp export accounted for over 470 million dollars in revenue, a 30 million dollar increase from 2013 (http://www.dfo-mpo.gc.ca/stats/facts-Info-14-eng.htm). It is also one of Canada's most imported seafood products. As such, the possibility that these diseases could be spread via importation or exportation always remains a great concern to the Canadian public.

In an effort to prevent the introduction or spread of disease, rigorous testing is required prior to shipment of crustaceans from areas where pathogens have previously been found. Further, areas importing crustaceans may wish to perform additional testing prior to accepting shipments (Walker and Winton, 2010). As such, assays for the detection of crustacean pathogens are an important part of any aquatic diagnostic laboratory portfolio. Sensitive molecular assays are often considered to be the best methods for the surveillance of samples destined for import or export (Hernandez-Herrera et al., 2007; Poulos et al., 2008; Sithigorngul et al., 2000; Sri Widada et al., 2004; Tang and Lightner, 1999). More specifically, quantitative PCR (qPCR) is often the preferred molecular assay, as the technique is very sensitive, inexpensive and the results can usually be obtained within a few hours to a few days from the reception of the samples (Andrade et al., 2007; Mendoza-Cano and Sanchez-Paz, 2013; Tang and Lightner, 2001; Tang et al., 2004).

In this study, we have focused on five crustacean pathogens listed as immediately notifiable in Canada: Taura Syndrome Virus (TSV), Yellow Head Virus (YHV), Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV), Infectious Myonecrosis Virus (IMNV) and the *Macrobrachium rosenbergii* Nodavirus (MrNV), which is now known to be the causative agent of White Tail Disease (WTD) (Sahul Hameed et al., 2004; Zhang et al., 2006). The first four of these viruses are known to infect a variety of penaeid shrimp species, while MrNV has only been found in the giant freshwater prawn *M. rosenbergii* (Table 1) (Lightner et al., 1997; Longyant et al., 2005; Robles-Sikisaka et al., 2002; Sri Widada et al., 2004; Sudhakaran et al., 2008; Tang and Lightner, 2001; Tang et al., 2005; Tang et al., 2003). While qPCR assays have previously been developed for some of these viruses, we opted to design new assays for each pathogen, for

a number of reasons. Firstly, we note that the amount of publically available genomic data for each viral species has drastically increased in recent years, and as such the ability to design specific primers has greatly increased (Dhar et al., 2010; Naim et al., 2015; NaveenKumar et al., 2013; Silva et al., 2014; Sittidilokratna et al., 2009). Further, the software tools used for the design of qPCR primers and probes also greatly improved permitting even further improvement to the development of sensitive and specific assays (Singh and Pandey, 2015). Finally, and perhaps more importantly, we have designed the assays to function as a suite of assays. In other words, although each assay is specific to the intended pathogen, all assays use common protocols and share many reagents. Having a suite of assays based on common protocols is a great asset to diagnostic labs, which will benefit from the reduced cost of reagents purchased in bulk, as well as simplified training for the analysts. Each test was designed around the use of TaqMan® fluorescent probe technologies, and the amplification efficiency, analytical sensitivity and specificity of the assays were assessed individually. We have compared our results to those listed in the OIE aquatic manual, and find that our assays perform comparatively well to the listed assays and have the added benefit of using a standard operating protocol.

MATERIALS AND METHODS

Nucleic acid extractions

Individual tissue samples from preserved Whiteleg shrimp (*Litopenaeus vannamei*) experimentally infected with IHHNV, YHV, TSV or IMNV were kindly provided by Dr. Donald Lightner (University of Arizona, USA), while *M. rosenbergii* post-larvae tissue infected with MrNv was obtained from Dr. A.S. Sahul Hameed (C. Abdul Hakeem College, India). RNA extractions were done using 10-20 mg of tissue following a standard RNA extraction protocol. Briefly, tissue samples were homogenized using a Fast Prep-24 Tissue and Cell Homogenizer (MP Biomedicals) and cells were lysed using TRI Reagent (Sigma-Aldrich). Chloroform was then added and samples were centrifuged at 12 000 x g for 15 minutes. The aqueous phase was transferred to new tubes containing an equal volume of isopropanol and samples were again centrifuged at 12 000 x g for 15 minutes. The resulting RNA pellet was washed once with ethanol and resuspended in 80 µL of 1 mM sodium citrate, containing 4 units of RNase inhibitor (Qiagen). RNA was quantified using a NanoDrop spectrophotometer (Thermo Scientific) and normalized to a 200 ng/µL final concentration.

Viral genome sequence alignments and primer and probe design

Viral sequences were obtained from the National Center for Biotechnology Information (NCBI), and aligned using Clustal X v.2.1 (Larkin et al., 2007), then viewed and edited in GeneDoc (Nicholas et al., 1997). Primers and probes used in this study were designed based on the alignments using the Primer Express software (Applied Biosystems, version 3.0.1). Primers were designed to have Tm values ranging between 55^{o}C and 62^{o}C (Table 2a), whereas PCR probes

were designed to have Tm values ranging between 68°C and 70°C. All probes were conjugated at the 5' end with a reporter dye and a minor groove binder non-fluorescent quencher (MGBNFQ) at the 3' end (Table 2b).

Reverse transcription and QPCR assays

Reverse transcription (RT) of RNA samples was carried out using random hexamers and the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturers' protocol, except water was added to a final volume of 20 µL. Up to 1 µg of total RNA was used as template for reverse transcription. RNA was first denatured at 95°C for 5 min and cooled down to 4°C before the RT mix was added. Thermocycling conditions consisted of an initial step at 25°C for 10 min, followed by 2 hours incubation at 37°C, and an enzyme inactivation step at 85°C for 5 min. The resulting cDNA was then diluted by half to a final volume of 40 µL. Quantitative PCR assays were carried out using 12.5 µL of the appropriate 2x PCR master mix (see Table 2a) and 2 µL of cDNA template. Primers were added to a final concentration of 0.32 µM, probes at a final concentration of 0.20 µM and bovine serum albumin to a concentration of 0.04%. Reactions were then brought to a final volume of 25µL using DEPC treated water. Cycling parameters consisted of an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of denaturation, 30s at 95°C, 30s annealing at the appropriate temperature (Table 2a) and 30s at 72°C for extension. PCR reactions were carried out in either Mx3000P (Agilent technologies) or StepOnePlus (Applied Biosystems) thermocyclers, and three fluorescence readings were taken at the end of every elongation step.

Plasmid construction

Each viral target sequence was amplified by end point PCR using the Platinum Taq (Life Technologies) DNA polymerase master mix supplemented with 0.32 µM of each primer and 2 µL (roughly 0.5 µg) of infected shrimp cDNA. The PCR amplicons were separated by agarose gel electrophoresis. DNA bands were excised from the gel and purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). Purified PCR products were then ligated using either an InsTAclone PCR Cloning Kit (Thermo Scientific) or the pGEM-T Vector System (Promega) following the manufacturers' protocol. Bacterial transformation was carried out using the TransformAid Bacterial Transformation Kit (Thermo Scientific) following the recommended protocol. White bacterial colonies were selected and grown overnight in LB media and plasmid DNA was extracted using the NucleoSpin Plasmid Miniprep Kit (Macherey-Nagel).

Reaction efficiencies and analytical sensitivity

To calculate reaction efficiencies, RNA was extracted from infected muscle tissues and reverse transcribed as described above. The resulting cDNA was then serially diluted one in ten, and RT-qPCR was performed. Cycle Thresholds (Ct) were obtained, plotted against the log of the relative concentration of the cDNA, and regression analysis was used to calculate the line slope and coefficient of determination. Reaction efficiency (E) is calculated using the formula E = -1 +

$10^{(-1/slope)}$. To determine the analytical sensitivity of the assays, the various target sequences were cloned into a plasmid and the plasmid concentrations were converted to target copies, using an average base pair weight of 650 Daltons. The formula used for this calculation was: $C_N = m / (650 * l / N_A)$, where "$C_N$" is the copy number, "m" is the mass (in grams) of the DNA present in the qPCR reaction, "l" is the length of the plasmid construction (in base pairs) and "N_A" is the Avogadro constant.

RESULTS

Optimization of RT-qPCR assays

In order to optimize all of the RT-qPCR assays, two separate primer pairs were originally designed for the detection of each viral pathogen. Preliminary gradient endpoint RT-PCR assays were performed with each primer pair to identify the best annealing temperature for each test. Amplicons were separated by electrophoresis on a 1% agarose gel. For every pathogen, we selected the primer pair that appeared to have the best mix of specificity and sensitivity based on qualitative observation of the gel (data not shown). These corresponded to primers identified in Table 2a. Fluorescent probes were then designed for the selected primer pairs in order to convert the end point RT-PCR assays to RT-qPCR (Table 2b). To further optimize the RT-qPCR assays, 2 different Taq master mix kits were compared for each test. The better performing kit, which was largely based on the Ct values and amplification plots, was then selected for each individual test (Table 3).

Standard curve and qPCR amplification efficiency assays

To determine the amplification efficiency of these newly developed RT-qPCR tests, we performed triplicate series of serial dilutions (diluted one in ten) for each test. Dilutions were prepared by mixing the extracted cDNA of the positive animal tissues with cDNA from negative shrimp tissues. Analysis of the results showed PCR efficiencies of 93.6% for IHHNV, 100.1% for IMNV, 98.0% for TSV, 92.2% for YHV and 97.2% for MrNV (Figures 1 to 5).

Analytical sensitivity and specificity of RT-qPCR assays

To evaluate the analytical sensitivity (also known as the lower limit of detection) of each assay, eight replicate qPCR reactions were performed for each test using serially diluted plasmid. These qPCR reactions were used to approximate the RT-qPCR, and permit us to calculate a target copy number. Tenfold serial dilutions (diluted with water) were first prepared from linearized plasmid constructs for each assay. The least concentrated plasmid dilution in which at least four of the PCR replicates (50%) gave positive results was considered to be the analytical sensitivity (or probability point) for that test, and the number of viral gene copies present in this dilution could then be calculated knowing the size of the plasmid constructs (Table 4). For IHHNV, as little as 13.4ag of plasmid was detected in 5/8 PCR replicates, which equals roughly 40 viral target

copies. For IMNV, 24.8ag of template was detected in 6/8 replicates, which equals to 80 copies. For TSV, as little as 2.86ag of plasmid could be detected in 5/8 replicates, which is equal to approximately 10 copies. For YHV, 56ag of template could be detected in 8/8 replicates, equalling roughly 170 viral copies. Finally, 56ag of MrNV template was detected in 8/8 replicates, which roughly equals 190 viral copies. The analytical specificity of each qPCR assay was also verified using genomic DNA of non-infected animals of the same species, except for MrNV, which was done using *Litopenaeus vannamei* genomic DNA, as we did not have access to any other *Macrobrachium rosenbergii* tissues (Table 5). In each case, no Ct values were obtained by qPCR, signifying that these assays were specific to the desired pathogen (data not shown). Although we did not have access to non-infected *M. rosenbergii* tissues or RNA, sequence analyses performed on NCBI nucleotide BLAST using the primer and probe sequences for the MrNV test did not show any significant similarity with the *M. rosenbergii* genome, and the primers do not amplify sequences from various other related crustaceans. Finally, no cross amplification has been observed using primers designed against one pathogen in a matrix containing target sequences from other pathogens (Table 5).

DISCUSSION

Every year, aquatic animal diseases cause major financial losses to the aquaculture industry. While countries experiencing persistent problems with specific pathogens will likely develop assays for surveillance and diagnosis of certain pathogens, countries where these pathogens have never existed may have few methods or resources to conduct testing for these, which may be introduced via importation. The purpose of this project was to develop a new sensitive and specific suite of qPCR assays able to detect various prawn and shrimp viruses that are currently listed by the OIE as notifiable diseases. We focused on five diseases for which molecular detection methods previously existed, and updated these tests to increase analytical sensitivity as well as to harmonize protocols. These tests will be used to support Canada's National Aquatic Animal Health Program.

While there is no absolutely universal classification system for viruses, one of the more accepted systems is that of David Baltimore (Baltimore, 1971). Briefly, it states that while viruses may use any number of nucleic acid types for their genomic material, all viruses must create mRNA in order to produce protein. Hence, in order to harmonize our protocols as much as possible, mRNA was chosen as the target for each virus, regardless of its genomic material. We initially designed at least two primer sets per pathogen, and using the best performing primer pair, converted the initial endpoint RT-PCR tests to RT-qPCR by designing a TaqMan® fluorescent probe.

Serial dilutions of cDNA were used to calculate reaction efficiencies. All of the assays designed have amplification efficiencies that fall within the generally accepted range of 90% to 110%. As our only source of pathogen RNA was infected tissues and that it is not possible to determine the

number of target copies from these tissues, we used plasmid DNA to determine the analytical sensitivity of our assays. Serial dilutions of the plasmid DNA were prepared using non-infected animal cDNA as the diluent instead of water to better emulate the conditions present when performing the assays in a diagnostic setting. While more often than not, the analytical sensitivity of an assay is defined as the lowest amount of target analyte than can be detected. We defined it as the lowest amount of target analyte where at least 50% of the PCR replicates show positive results. Given that at low concentration analyte would follow a Poisson distribution (Welzel et al., 2006), it is common that qPCR reactions are not repeatable at very low concentrations of analyte. As such, it is also common for analytical sensitivity to be defined as the lowest concentration at which an analyte is detected. Given that these assays will be used in a diagnostic context, we chose to call the limit of detection the point where at least 50% of the replicates gave a positive result. In the case of IHHNV, the lowest viral copy number our test detected was 40, which is in the same order as the 10 copies that were needed for detection in a study by Tang and Lightner (2001). Note that this previous study used the more classical definition of analytical sensitivity, and did not perform as many replicate qPCR reactions. By comparison, our assay was able to detect IHHNV from only 4 genomic copies in two out of eight replicate samples, giving it a slightly lower detection limit, in those terms. Importantly, it must be noted that at these low levels of detection, there are a number of confounding factors, not directly related to assay performance, which can lead to errors in the calculations of lower detection limits. For example, amplification kinetics related to DNA conformation may play a role. While both our study and that of Tang and Lightner used plasmid DNA to determine assay sensitivity, our study used linearized plasmid DNA. It is known that the kinetics of DNA amplification can differ greatly between these two DNA conformations. More specifically, it has been shown that supercoiled plasmid has a slight inhibitory effect on qPCR. (Hou et al., 2010). Further, and perhaps more importantly, the fluidics of micro-pipetting such low concentrations of template are such that, despite the use of high precision liquid handling systems, it is unlikely that the calculated number of target molecules in a reaction correspond to the actual number of molecules (Lievens et al., 2012). In short, we believe that analytical sensitivities at this low level can be considered equivalent unless they differ by at least an order of magnitude. Similar observations can be made for the other assays. For example, Andrade *et al* (2007) showed that between 10 and 100 copies of IMNV was all that was needed for detection by their real-time RT-PCR assay, which is comparable to our limit of detection of 80 copies. Tang *et al* (2004) designed a TaqMan assay with a limit of detection of 100 copies of the TSV genome, compared to our assay which could detect as little as ten copies. As for MrNV, the OIE refers to a study conducted by Hernandez-Herrera *et al* (2007) who have developed an RT-qPCR assay based on SYBR Green technology, which is generally considered to be less specific than TaqMan assays. Unfortunately, the authors did not determine the analytical sensitivity of their assay, so we cannot compare results. Currently, there is no reference RT-qPCR test for YHV listed in the OIE's Manual of Diagnostic Tests for Aquatic Animals, although a recent paper describes such an assay (Soowannayan et al., 2013). Unfortunately, as with many published papers, no

analytical details are given, thus accentuating the importance of the development of a reliable RT-qPCR assay for the detection of YHV, as done in the current study.

The bench validation we conducted provides clear values for amplification efficiency, analytical sensitivity and specificity of each of our assays, making them suitable for disease testing. These assays will be transferred to DFO National Aquatic Animal Health Laboratories and will be available for use for screening purposes in events of importation or exportation requests, or disease outbreak suspicion in penaeid shrimp.

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Table 1 – Viral pathogen types and hosts species

Virus name	Baltimore Classification	Susceptible host species*	Geographical distribution
Infectious Hypodermal and Haematopoietic Necrosis Virus (IHHNV)	**Group II** Single-stranded DNA viruses	Most penaeid species, especially *Penaeus monodon, P. vannamei,* and *P. stylirostris*.	Worldwide, with the possible exception of the North American East-Coast.
Infectious Myonecrosis Virus (IMNV)	**Group III** Double-stranded RNA viruses	*Penaeus vannamei*	North-eastern Brazil and South-East Asia
Macrobrachium rosenbergii Nodavirus (MrNV)	**Group IV** Single-stranded RNA viruses - Positive-sense	*Macrobrachium rosenbergii*	French West Indies, China, India, Chinese Taipei, Thailand and Australia.
Taura Syndrome Virus (TSV)	**Group IV** Single-stranded RNA viruses - Positive-sense	*Penaeus setiferus, P. schmitti, P. monodon, P. chinensis, P. japonicus, P. aztecus, P. duorarum and Metapenaeus ensis*	Americas and South-East Asia
Yellow Head Virus** (YHV)	**Group IV** Single-stranded RNA viruses - Positive-sense	*Penaeus monodon, P. vannamei, P. japonicus, P. merguiensis, P. stylirostris, P. setiferus, P. esculentus, P. aztecus, P. duorarum,Metapenaeus ensis, Palaemon styliferus, Euphasia superba, Metapenaeus bennettae, Macrobrachium sintangense, Palaemon serrifer, Ascetes* spp.	China, Chinese Taipei, India, Indonesia, Malaysia, the Philippines, Sri Lanka, Thailand, Vietnam, Australia, Mozambique, Mexico

* The susceptible host species listed here include only species whose natural populations have been infected. In most cases, the listed viruses have been shown infect a much larger number of crustacean species in laboratory challenges.

** Yellow Head disease is associated with six genotypically distinct viruses, which are considered as a single viral species called the Gill-associated virus. Genotype I of this species is referred to as Yellow Head Virus, and is the only known agent of Yellow Head disease. The additional five related viruses are found in the gills of healthy penaeid species, and have not been associated with disease.

Table 2a. Primer sets and cycling conditions for amplification of pathogen nucleic acids

Primer name	Primer sequence (5' -> 3')	Size of amplicon	Annealing Temperature (°C)	PCR mastermix
IHHNV 828F IHHNV 898R	AGC TAC AAT CCT CGC CTA TYT GG CAA GTA CCG TAG TCG CTT CAG CTT	71bp	58	TaqMan Universal
IMNV 247F IMNV 313R	GCC CTG CCA ACT GTA AAT TTG TGT CGC CAA GTG TGA AAT CG	67bp	61	TaqMan Gene Expression
TSV 777F TSV 836R	CGG CTT CAA TTA TCC AGC AGA T GGA GCA CGC GTT ACT GAA A	60bp	55	TaqMan Universal
MrNV 148F MrNV 209R	TTA CAC ATG GAC CAC GAC TCA TTC ATA AGG TCC GAT TAC CAC ATA	62bp	58	TaqMan Gene Expression
YHV 311F YHV 375R	GGA CCA CAG AAC AAA CTC TCM AA AGG GTG TGG TAT GGG AAT GG	65bp	58	TaqMan Universal

Table 2b. Reporter probes and target sequence for detection of pathogen nucleic acids

Probe name	Probe sequence (5' ->3')	Target sequence
IHHNVP852F	(FAM) AGT TAC CTT TGC TGC CAG AG (MGB)	Non-Structural protein 2 (as per AY355307.1)
IMNVP270F	(VIC) TGA CTA TCT TGT ATC CTG GGC (MBG)	Coat protein (ORF 1) (as per AY570982.2)
TSVP800F	(FAM) TTC CTG AGG AGC CCA CT (MGB)	Capsid Protein 2 (as per FJ876500.1)
MrNV 170-P	(FAM) CCG CAG ATT AGC TTC (MGB)	RNA-dependent RNA polymerase (as per FJ751226.1)
YHVP335F	(VIC) ATG TAC ACT GAC AAC ATC (MGB)	ORF 1B (as per EU785034.1)

Table 3 – Selection of enzyme mix

Pathogen		Universal	Gene expression
	Dilution	Ct value	Ct value
IHHNV	1/25	**24.53**	24.92
	1/125	**26.94**	27.42
	1/625	**33.49**	33.31
	1/3125	**36.63**	36.43
IMNV	1/25	18.20	**17.76**
	1/125	21.31	**20.27**
	1/625	28.63	**27.34**
	1/3125	31.76	**30.40**
TSV	1/25	**16.13**	16.72
	1/125	**17.90**	18.77
	1/625	**20.70**	21.03
	1/3125	**23.46**	23.73
YHV	1/25	**23.72**	24.15
	1/125	**26.59**	26.76
	1/625	**32.89**	33.67
	1/3125	**35.60**	37.77
MrNV	1	13.61	**14.20**
	1/10	16.95	**17.50**
	1/100	21.58	**21.59**
	1/1000	30.99	**30.42**
	1/10000	39.73	**36.76**

Table 4. Analytical sensitivity of the assays

Pathogen	Detection limit (OIE)	Absolute detection limit
IHHNV	40 copies	4 copies
IMNV	80 copies	8 copies
TSV	10 copies	1 copy
YHV	170 copies	17 copies
MrNV	190 copies	19 copies

Table 5. Analytical specificity of the assays

Assay	Host	IHHNV	IMNV	MrNV	TSV	YHV
IHHNV	-	+	-	-	-	-
IMNV	-	-	+	-	-	-
MrNV	-	-	-	+*	-	-
TSV	-	-	-	-	+	-
YHV	-	-	-	-	-	+

* The analytical specificity of the MrNV qPCR assay could not be evaluated using non-infected genomic DNA of *M. rosenbergii,* because no other samples of this species could be obtained. This assay's specificity was instead done using *Litopenaeus vannamei* genomic DNA.

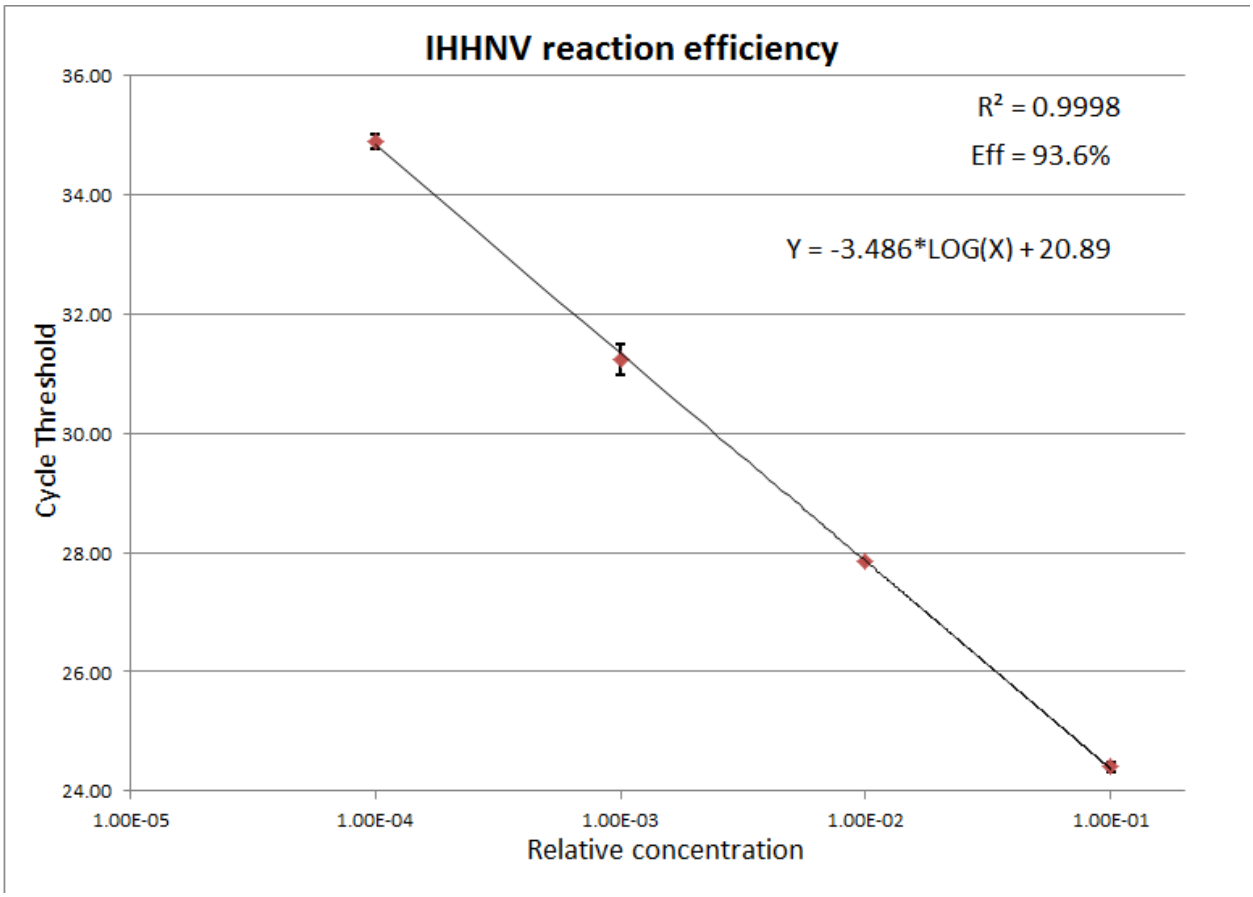

Figure 1 – RT-qPCR reaction efficiency of the IHHNV assay. RNA was extracted from a muscle tissue infected with the IHHN virus, and reverse transcribed. The resulting cDNA was then serially diluted, 1/10, and RT-qPCR was performed. Cycle Thresholds were obtained and plotted against the relative concentration of the cDNA, and regression analysis was used to calculate the line slope and coefficient of determination. Reaction efficiency (E) is calculated using the formula $E = -1 + 10^{(-1/slope)}$.

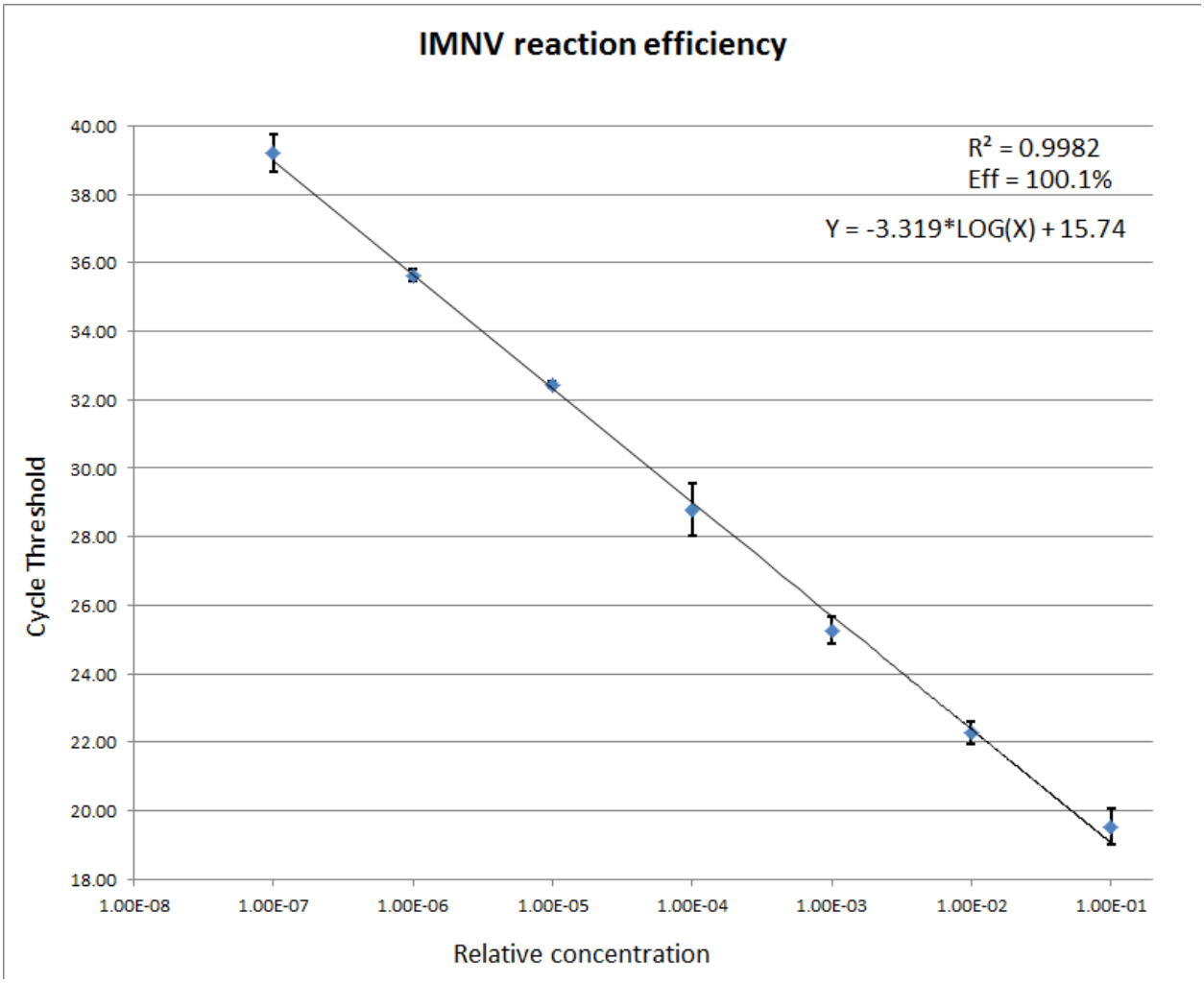

Figure 2 – RT-qPCR reaction efficiency of the IMNV assay. RNA was extracted from a muscle tissue infected with the IMN virus, and reverse transcribed. The resulting cDNA was then serially diluted, 1/10, and RT-qPCR was performed. Cycle Thresholds were obtained and plotted against the relative concentration of the cDNA, and regression analysis was used to calculate the line slope and coefficient of determination. Reaction efficiency (E) is calculated using the formula $E = -1 + 10^{(-1/slope)}$.

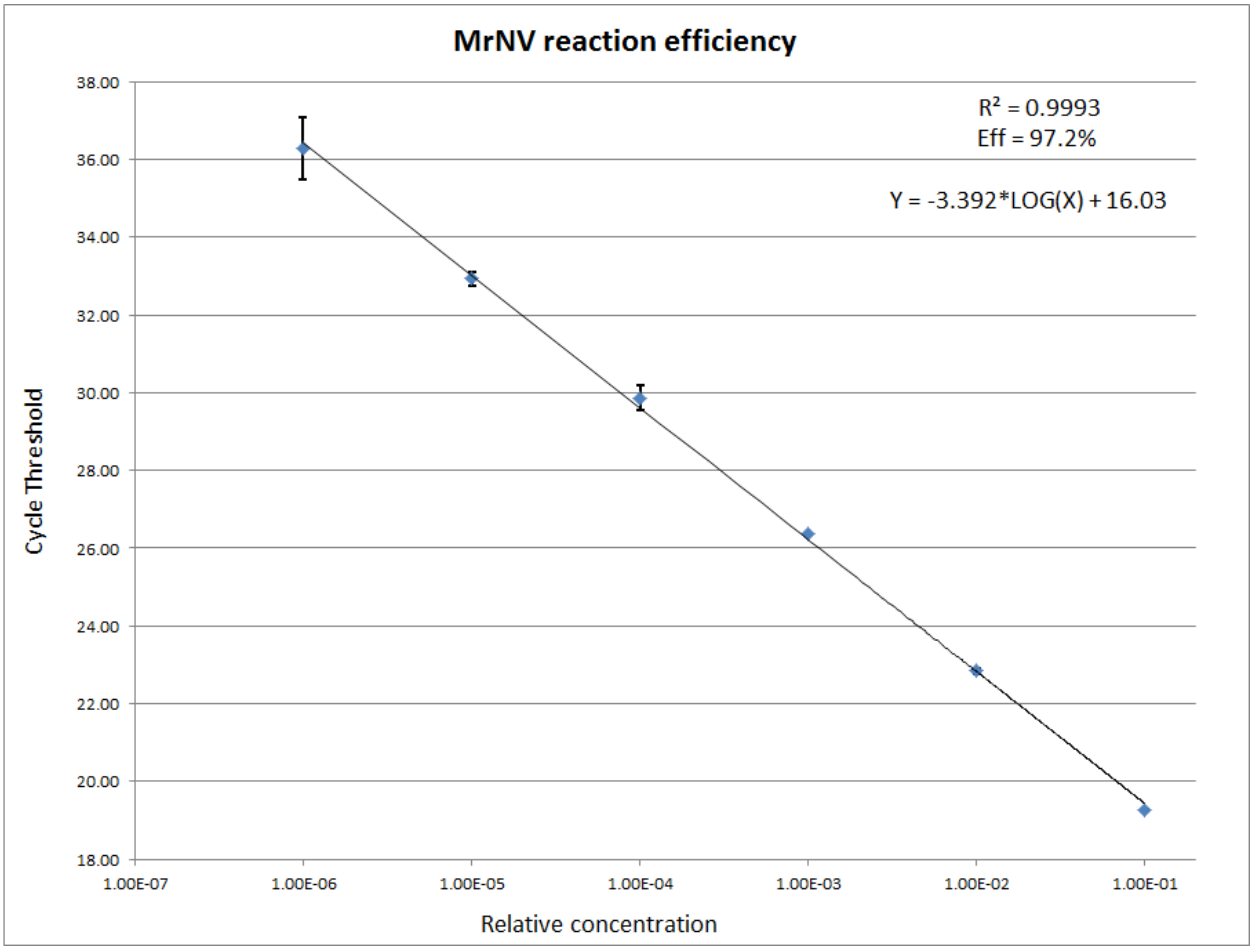

Figure 3 – RT-qPCR reaction efficiency of the MrNV assay. RNA was extracted from a muscle tissue infected with the MrN virus, and reverse transcribed. The resulting cDNA was then serially diluted, 1/10, and RT-qPCR was performed. Cycle Thresholds were obtained and plotted against the relative concentration of the cDNA, and regression analysis was used to calculate the line slope and coefficient of determination. Reaction efficiency (E) is calculated using the formula $E = -1 + 10^{(-1/slope)}$.

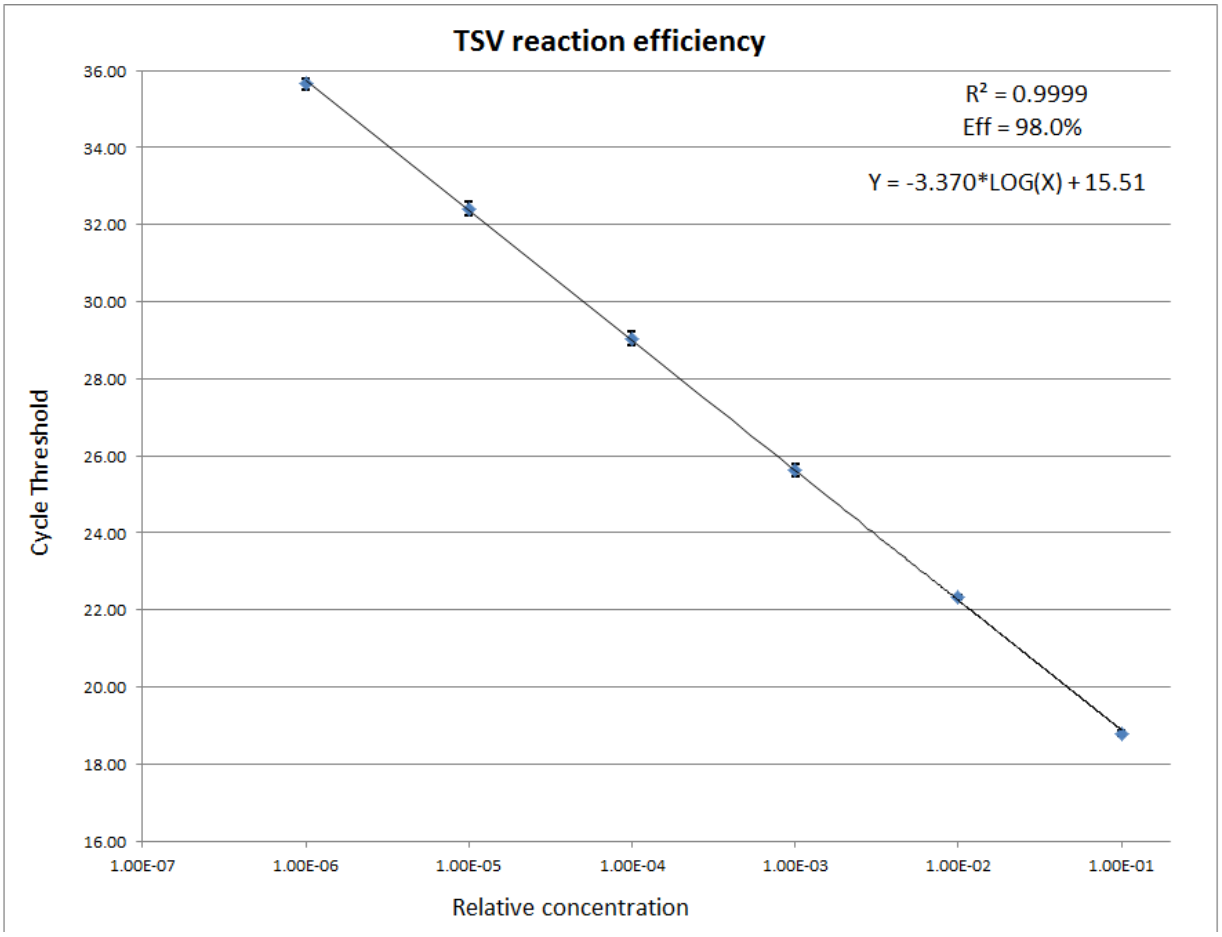

Figure 4 – RT-qPCR reaction efficiency of the TSV assay. RNA was extracted from a muscle tissue infected with the TS virus, and reverse transcribed. The resulting cDNA was then serially diluted, 1/10, and RT-qPCR was performed. Cycle Thresholds were obtained and plotted against the relative concentration of the cDNA, and regression analysis was used to calculate the line slope and coefficient of determination. Reaction efficiency (E) is calculated using the formula $E = -1 + 10^{(-1/slope)}$.

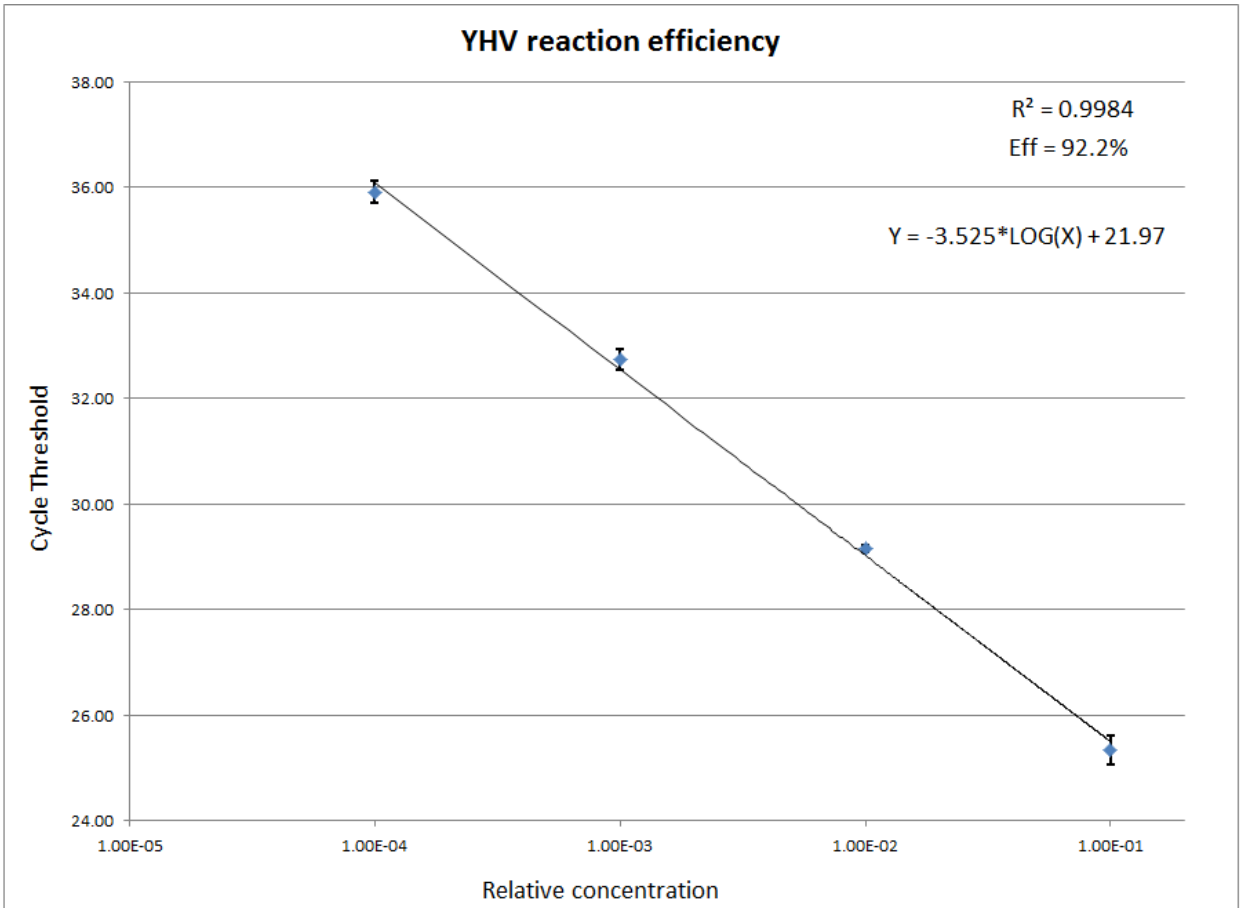

Figure 5 – RT-qPCR reaction efficiency of the YHV assay. RNA was extracted from a muscle tissue infected with the YH virus, and reverse transcribed. The resulting cDNA was then serially diluted, 1/10, and RT-qPCR was performed. Cycle Thresholds were obtained and plotted against the relative concentration of the cDNA, and regression analysis was used to calculate the line slope and coefficient of determination. Reaction efficiency (E) is calculated using the formula $E = -1 + 10^{(-1/slope)}$.