Vol. 129: 193–198, 2018
https://doi.org/10.3354/dao03242

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published August 14

Partial validation of a TaqMan real-time quantitative PCR assay for the detection of *Panulirus argus* virus 1

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ABSTRACT: The Caribbean spiny lobster *Panulirus argus* supports important fisheries throughout the greater Caribbean and is also the only known host for the pathogenic virus *Panulirus argus* virus 1 (PaV1). While discovered nearly 2 decades ago, gaps still exist in our knowledge of PaV1, such as the dose required to establish infection and its viability outside of the host. To help answer such questions and to enhance diagnostic capabilities, we developed a TaqMan real-time quantitative polymerase chain reaction (qPCR) assay for PaV1. Of the advantages offered by qPCR, one of the most important benefits is its ability to accurately quantify viral DNA copies in a clinical sample. The qPCR assay was found to be efficient (mean ± SD: 99.19 ± 4.67 %) and sensitive, detecting as few as 10 copies of PaV1 plasmid DNA. Its diagnostic sensitivity and specificity determined using a set of 165 lobster samples (138 from Florida, USA, and 27 from across the Caribbean) were 100 and 84 %, respectively. The qPCR assay should thus prove useful as a research tool and for detecting and quantifying PaV1 infection severity in Caribbean spiny lobsters.

KEY WORDS: *Panulirus argus* virus 1 · Quantitative PCR · Lobster · Diagnostics · Sensitivity · Specificity

INTRODUCTION

Panulirus argus virus 1 (PaV1) is a pathogenic virus infecting the Caribbean spiny lobster *Panulirus argus*. Since its discovery in 2000, much has been done to understand its epidemiology (Shields & Behringer 2004, Butler et al. 2008, Lozano-Álvarez et al. 2008, Moss et al. 2013), ecology (Behringer et al. 2006, Behringer & Butler 2010, Anderson & Behringer 2013), and impacts on *P. argus* fishery populations (Cruz Quintana et al. 2011, Behringer et al. 2012, Briones-Fourzán et al. 2012, Candia-Zulbarán et al. 2012, Huchin-Mian et al. 2013). Although commonly detected in commercial trap- and casita-based fisheries across the Caribbean, the long-term effects of PaV1 on lobster landings remain equivocal (Behringer et al. 2012, Candia-Zulbarán et al. 2012).

PaV1-infected lobsters display behavioral abnormalities such as occasional tremors and lethargy that may manifest as individuals being unable to right themselves, and they can also exhibit gross signs

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including milky hemolymph and/or carapace discoloration (Shields & Behringer 2004). Healthy lobsters avoid PaV1-infected conspecifics, causing an increase in den numbers with solitary occupants (Behringer et al. 2006). Infected hemocytes (e.g. hyalinocytes and semi-granulocytes) and spongy connective tissue (Shields & Behringer 2004, Li et al. 2006) display microscopic lesions typified by karyomegaly, margination of condensed chromatin, and faint eosinophilic nuclear inclusions (Shields & Behringer 2004). Cytopathic effects in hemocytes have been used to quantify virus infectivity in tissue culture infectious dose 50% endpoint ($TCID_{50}$) assays (Li & Shields 2007). By transmission electron microscopy (TEM), arrays of naked polygonal PaV1 nucleocapsids (~180 nm in diameter) with spherical electron-dense DNA cores (~118 nm in diameter) assemble in the nucleus of infected cells (Shields & Behringer 2004).

Despite it being investigated extensively, PaV1 remains an unclassified DNA virus. While speculated to be a nucleocytoplasmic large DNA virus (T. B. Waltzek et al. unpubl. data), only 2 short genome sequences have been published (Li et al. 2006, Montgomery-Fullerton et al. 2007). One of these sequences has been used to develop a fluorescence *in situ* hybridization test, which combined with histopathology and TEM data has shown PaV1 to replicate in cells in and around the hepatopancreas, hindgut, foregut, gill, heart, skin, nerves, and ovaries (Li et al. 2006). A region of the PaV1 genome encoding a hypothetical protein has been used to develop a specific and sensitive endpoint PCR assay (Montgomery-Fullerton et al. 2007, Huchin-Mian et al. 2009, Moss et al. 2012, 2013). To expand the diagnostic tests available for PaV1, we report a rapid and cost-effective TaqMan real-time quantitative (q)PCR assay designed to accurately detect and quantify PaV1 DNA loads in infected lobsters.

MATERIALS AND METHODS

TaqMan qPCR primers and probe

A set of 61 PaV1 hypothetical protein coding sequences detected in *Panulirus argus* from 8 regions in the Caribbean Sea (Moss et al. 2012, 2013) and available in GenBank were aligned in MAFFT (Katoh & Toh 2008). A consensus nucleotide sequence was then generated using BioEdit 7.2.6.1 (Hall 1999). PrimerExpress v2.0 (Applied Biosystems) was used to design PaV1 PCR assay primers and a [6FAM]-MGB/NFQ-labeled TaqMan hydrolysis probe (Table 1). Default settings were used in each software and a probe substantially shortened by use of the MGB fluorescence quencher was employed to maximize test specificity and signal-to-noise ratio.

qPCR

Reactions (20 µl) contained 0.9 µM each primer, 0.25 µM probe, 4 µl nucleic acid template (≤100 ng total DNA), 10 µl universal qPCR mix (TaqMan® Fast Universal PCR Master Mix 2×, Applied Biosystems), and 3 µl molecular grade water and were amplified in 96-well polypropylene plates (Olympus Plastics, Genesee Scientific) sealed with a 50 µm polyolefin film cover (ThermalSeal RTS, Excel Scientific). DNA templates and standards comprising serial 10-fold dilutions of linearized plasmid DNA (10^7 to 1 copy) containing the PaV1 hypothetical protein sequence were amplified in triplicate. Each sample was also amplified using a eukaryote 18S rRNA endogenous control assay (Applied Biosystems Assay ID Hs-99999901_s1). All liquid handling was performed by a QIAgility instrument (Qiagen). Thermal cycling (95°C for 20 s followed by 40 cycles at 95°C for 3 s and 60°C for 30 s) used a 7500 Fast Real-Time PCR

Table 1. Sequences and characteristics of the primers and probe used in the *Panulirus argus* virus 1 (PaV1) TaqMan qPCR assay and an endpoint PCR assay

Primer/ probe	Sequence (5′–3′)	Melting temp. (°C)	Position in gene (5′–3′)	Amplicon size (nt)
45aF	TTCCAGCCCAGGTACGTATC	62.3	45–64	499[a]
543aR	AACAGATTTTCCAGCAGCGT	58.4	524–543	
PaV1F	CGTTGTACGGAATCGTTATTAAAGC	61.3	256–280	69
PaV1R	GACACGACCAATTGAAGAAAAACTAC	61.4	299–324	
PaV1P	6FAM-CCCGTGATGCTTGC-MGB/NFQ	52.9	284–297	

[a]A 499 bp DNA product amplified using the endpoint PCR primers 45aF and 543aR (Montgomery-Fullerton et al. 2007) was cloned into a plasmid used to determine PaV1 qPCR performance

System (Applied Biosystems). A threshold cycle (Ct) was calculated and interpreted as a positive result for samples if the ROX (passive reference dye)-normalized FAM signal exceeded the threshold assigned by the Applied Biosystems software.

qPCR characteristics

The PaV1 qPCR slope, *y*-intercept, correlation coefficient (R^2), efficiency, dynamic range, analytical sensitivity, repeatability, reproducibility, and analytical specificity were estimated from 21 independent amplifications of the serially diluted linearized plasmid DNA. Test efficiency was calculated as $10^{-1/\text{slope}} - 1$ (Bustin et al. 2009) by the Applied Biosystems software. To measure qPCR assay performance, inter-assay variability (reproducibility) and intra-assay variability (repeatability) were determined using the percent coefficient of variation (CV% = [standard deviation/mean] × 100%) calculated using the standard deviation of each Ct within (repeatability) or among (reproducibility) the 21 tests.

The ability of the qPCR to detect only PaV1 (analytical specificity) was assessed by testing a set of 116 PaV1-positive samples (see below) and DNA extracted from clinical samples infected with double-stranded DNA iridoviruses from the genera *Lymphocystivirus* (lymphocystis disease virus, LCDV), *Megalocytivirus* (infectious spleen and kidney necrosis virus, ISKNV), and *Ranavirus* (frog virus 3, FV3) (Table 2). The LCDV sample originated from a copperband butterflyfish *Chelmon rostratus* displaying gross proliferative fin lesions and was LCDV-positive by PCR (Hanson et al. 2006). The ISKNV and FV3 DNA samples were as described previously (Tan et al. 2004, Subramaniam et al. 2016). The PaV1 qPCR was also tested using DNA extracted from a penaeid shrimp containing white lesions typical of infection with the nimavirus white spot syndrome virus (WSSV) confirmed to be WSSV-positive by PCR (Liang et al. 2011), and from tissue of a European green crab infected with *Carcinus maenas* virus 1 (CmV1) kindly provided by Dr. Grant Stentiford.

qPCR diagnostic sensitivity and specificity

A total of 165 samples of either hemolymph or leg tissue were acquired from juvenile (<76 mm carapace length, CL) or adult (≥76 mm CL) *P. argus* collected throughout the Caribbean Sea in 2008, 2009, or 2015 (Table 2). Insulin syringes (27 G × 5/8 1cc) were used to draw 0.1–0.2 ml hemolymph from the proximal joint of the fifth periopod. Alternatively, leg tissue was sampled when hemolymph could not be collected for logistical reasons (e.g. leg sinus too small for phlebotomy or when syringes were not available at some locations). Each sample was transferred to a 1.5 ml microcentrifuge tube containing 0.9 ml 95% ethanol and stored at −20°C until processed at the University of Florida Aquatic Pathobiology Laboratory (Gainesville, FL, USA).

DNA was extracted using either a DNeasy Blood and Tissue Kit (Qiagen) or using a Quick-gDNA MicroPrep Kit (Zymo Research) with the following protocol modifications. Hemolymph was removed from the −20°C freezer and then incubated at room temperature for 10 min. Next, hemolymph was centrifuged at 14 950 × *g* for 1 min. The ethanol was decanted carefully, and the pellet was air dried for 1 h. After 1 h, 25 µl of hemolymph were transferred to a clean microcentrifuge tube. For leg tissue samples, the 95% ethanol was poured off and the leg was macerated using a surgical blade. A 200 µl volume of lysis buffer was then added to either sample type

Table 2. Origins of *Panulirus argus* virus 1 (PaV1) clinical samples and other double-stranded DNA virus samples used to validate PaV1 TaqMan qPCR and endpoint PCR (EP-PCR) assay performance; NT: not tested in the present study; WSSV: white spot syndrome virus; CmV1: *Carcinus maenas* virus 1; ISKNV: infectious spleen and kidney necrosis virus; FV3: frog virus 3; LCDV: lymphocystis disease virus

Origin	Virus	Sample number	qPCR +ve	EP-PCR +ve	Reference
Florida Keys, USA	PaV1	138	138	131	This study
Dominican Republic	PaV1	6	6	6	Moss et al. (2013)
Puerto Rico	PaV1	8	8	8	Moss et al. (2013)
Mexico	PaV1	3	3	3	Moss et al. (2013)
Belize	PaV1	4	4	4	Moss et al. (2013)
Panama	PaV1	1	1	1	Moss et al. (2013)
Cuba	PaV1	1	1	1	Moss et al. (2013)
Bahamas	PaV1	4	4	4	Moss et al. (2013)
Arizona, USA[a]	WSSV	1	0	0	D. Lightner (gift)
UK	CmV1	1	0	NT	G. Stentiford (gift)
Midwest, USA	ISKNV	1	0	NT	Subramaniam et al. (2016)
Missouri, USA	FV3	1	0	NT	Waltzek et al. (2014)
Florida, USA	LCDV	1	0	NT	T. B. Waltzek (unpubl.)

[a]Tissue from shrimp infected experimentally with WSSV

(hemolymph or leg) before the tube was pulse vortexed and centrifuged briefly. A pestle was then used to manually disrupt each leg tissue sample. After 20 min at room temp, tubes were pulse vortexed and centrifuged. A NanoDrop 2000 Spectrophotometer (Thermo Scientific™) was used to estimate DNA concentration and purity prior to DNA storage at −20°C. DNA was tested using both the PaV1 qPCR and the PaV1 endpoint PCR as described previously (Montgomery-Fullerton et al. 2007 for primers, Moss et al. 2012 for thermal cycling conditions). In total, 116 known PaV1-positive and 49 PaV1-negative samples were used to estimate the diagnostic sensitivity and specificity of the qPCR assay (Table 2).

RESULTS

PaV1 TaqMan real-time qPCR primer and probe specificity

From the PrimerExpress v2.0 (Applied Biosystems) analysis, a TaqMan real-time qPCR primer/probe set was selected that amplified a 69 bp region of the PaV1 genome. *In silico* examination of the specificity of the PaV1 qPCR primer and probe sequences (Table 1) with the 61 PaV1 strain sequences available in GenBank identified 3 with nucleotide mismatches at either nt positions 37 or 38 within the PaV1P probe target sequence and 1 with a single mismatch at nt position 53 within the PaV1R reverse primer target sequence (Fig. 1).

PaV1 qPCR amplification characteristics

Based on 21 independent PaV1 qPCR runs, means ± SD calculated for various amplification parameters were: slope = −3.35 ± 0.12, *y*-intercept = 38.25 ± 1.06, R^2 = 0.989 ± 0.003, and efficiency = 99.19 ± 4.67%. Given that a Ct value was generated for the 1 plasmid DNA copy dilution in 65% (41/63) of the tests performed, the qPCR reliable limit of detection (analytical sensitivity) was specified as the previous dilution (i.e. 10 copies of linearized PaV1 plasmid DNA) for which all tests generated a Ct value (Table 3). The assay CV ranged from 1.96 to 3.69% for inter-assay variability (reproducibility) and from 0.04 to 1.74% for intra-assay variability (repeatability) (Table 3). The PaV1 qPCR assay reproducibility and repeatability were within acceptable limits (<5%) (Marancik & Wiens 2013).

The specificity of the PaV1 qPCR primers and probe assumed from the *in silico* analysis (Fig. 1) was confirmed by the assay detecting all of the 116 samples determined previously to be PaV1-positive by endpoint PCR. None of the other DNA viruses tested (ISKNV, LCDV, FV3, WSSV, and CmV1) generated a Ct value (Table 2), and the 18S rRNA internal control was positive in all samples tested.

Estimation of PaV1 qPCR diagnostic sensitivity and specificity

In testing the 116 samples determined to be PaV1-positive and 49 samples determined to be PaV1-negative when tested using a PaV1 endpoint PCR, the PaV1 TaqMan real-time qPCR generated a Ct value for all 116 (100% diagnostic sensitivity) PaV1-positive samples and for 8 of the 49 (41/49 = 84% diagnostic specificity) PaV1-negative samples (Table 2).

DISCUSSION

Here we report a TaqMan real-time qPCR assay to detect PaV1 DNA in its primary host, Caribbean

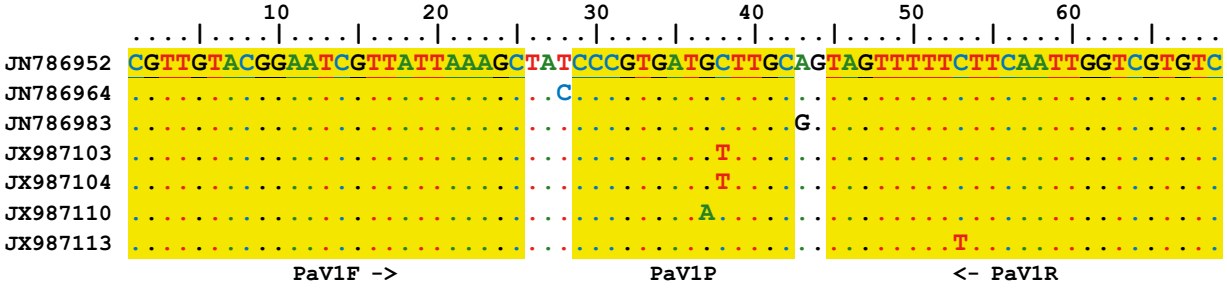

Fig. 1. *Panulirus argus* virus 1 (PaV1) qPCR amplicon sequence (69 bp) in the 499 bp hypothetical gene region used for *in silico* qPCR primer and probe design. A total of 61 PaV1 strain sequences available in GenBank were aligned (accession nos. JN786952 to JN786984, Moss et al. 2012; JX987103 to JX987130, Moss et al. 2013), but only 6 differing from the reference sequence JN786952 in the qPCR amplicon region are shown. The sequences targeted by the qPCR forward (PaV1F) and reverse (PaV1R) primers and probe (PaV1P) are shaded and underlined

Table 3. Inter-assay (reproducibility) and intra-assay (repeatability) variability of the *Panulirus argus* virus 1 (PaV1) TaqMan qPCR assay. To demonstrate reproducibility, samples were tested in triplicate in 21 independent qPCR runs performed over several days. To demonstrate repeatability, data obtained in a single representative qPCR run and using plasmid DNA (pDNA) are shown. Ct: threshold cycle number; CV: coefficient of variation

pDNA copy number	Inter-assay reproducibility Mean Ct ± SD	CV (%)	No. of +ve tests (n = 63)	Intra-assay repeatability Mean Ct ± SD	CV (%)	No. of +ve tests (n = 3)
10^7	15.3 ± 0.30	1.96	63	15.7 ± 0.05	0.32	3
10^6	18.0 ± 0.46	2.56	63	18.4 ± 0.06	0.33	3
10^5	21.2 ± 0.54	2.55	63	21.6 ± 0.05	0.23	3
10^4	24.7 ± 0.63	2.55	63	25.0 ± 0.01	0.04	3
1000	28.1 ± 0.69	2.46	63	28.2 ± 0.05	0.18	3
100	31.4 ± 1.10	3.50	63	31.6 ± 0.22	0.70	3
10	35.0 ± 1.00	2.86	63	35.0 ± 0.54	1.54	3
1	38.2 ± 1.41	3.69	41	38.5 ± 0.67	1.74	2

spiny lobsters. An *in silico* analysis detected only single nucleotide mismatches in either the PaV1 qPCR primer or probe target sites in 4 of 61 sequences from PaV1 strains detected in lobsters collected from across the Caribbean Sea (Moss et al. 2012, 2013). The assay exhibited a high correlation coefficient and was highly efficient, sensitive, specific, repeatable, and reproducible. It detected 10 copies of linearized PaV1 plasmid DNA with 100% reliability and did not amplify double-stranded DNA of other crustacean viruses, confirming its high analytical sensitivity and specificity. The PaV1 qPCR was also positive for all 116 DNA samples (100% diagnostic sensitivity) determined previously to be PaV1-positive using an endpoint PCR (Montgomery-Fullerton et al. 2007 for primers, Moss et al. 2012 for thermal cycling conditions), but was PaV1-positive for 8 of 49 DNA samples determined to be PaV1-negative using the endpoint PCR (84% diagnostic specificity). Overall, the analytical and diagnostic performance of the PaV1 qPCR adequately meets criteria for diagnostic PCR assays outlined by the World Organization for Animal Health (OIE 2016).

In comparison to the PaV1 endpoint PCR (Montgomery-Fullerton et al. 2007, Moss et al. 2012), the PaV1 TaqMan real-time qPCR described here offers several advantages including (1) speed and no requirement to detect a DNA band using agarose gel electrophoresis, (2) the ability to accurately quantify PaV1 DNA loads, and (3) increased analytical sensitivity and specificity, with the only disadvantages being access to a compatible thermal cycling instrument and the endpoint PCR generating an amplicon that can be sequenced for use in epidemiological and phylogenetic investigations (Moss et al. 2013).

Of the 49 samples determined to be PaV1-negative by endpoint PCR, 8 generated high Ct values (mean ± SD = 37.11 ± 1.03) using the PaV1 qPCR assay equivalent to <10 copies per 4 µl of the control linearized PaV1 plasmid DNA. Whether these represented false positives, either due to the primer and probe binding a non-specific target or to sample cross-contamination, or low-level PaV1-positive samples missed by the endpoint PCR but detected by the PaV1 real-time qPCR, is not known. However, agarose gel analysis of endpoint PCR amplifications of the linearized PaV1 plasmid DNA dilution series identified a DNA product in only 1/6 (16.7%) performed with the 1 copy dilution (data not shown), as opposed to 41/63 (65.1%) performed with the same dilution using the PaV1 qPCR assay. Moreover, the limit of detection of the endpoint PCR was reported to be 0.02 fg PaV1 DNA μl^{-1} of a PCR product of ~500 bp (Moss et al. 2012). Based on the amplicon mass, 0.02 fg would equate to 37 DNA copies (Thermo Fisher Scientific 2017). These data confirm that the PaV1 qPCR assay has a lower limit of detection than the endpoint PCR, and suggest that its diagnostic specificity might not be compromised relative to this test.

Applications for the PaV1 TaqMan real-time qPCR assay will include (1) determining the tissue distribution of PaV1 and how viral loads progress in various tissues over time following infection as examined during WSSV infection of giant tiger prawn *Penaeus monodon* (Jeswin et al. 2015), (2) detecting viral DNA in seawater samples for environmental DNA (eDNA) monitoring of PaV1, and (3) surveying PaV1 prevalence among disparate spiny lobster populations in the Caribbean.

Acknowledgements. We thank Mark Butler, Angelo Jason Spadaro, Joshua Anderson, and Jack Butler for assisting with sample collection and Linda Archer, Natalie Stilwell, Patrick Thompson, Jason Ferrante, Galaxia Cortés-Hinojosa, Gabriel Diaz, Anna Swigris, and Jaime Haggard for molecular testing support. The project was funded by a Florida Sea Grant Scholars program grant (A.S.C.), a University of Florida Opportunity Seed Fund grant (D.C.B. and T.B.W.), and a National Science Foundation – Biological Oceanography grant OCE-0928398 (D.C.B.).

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Editorial responsibility: Jeff Cowley, St. Lucia, Queensland, Australia

Submitted: September 14, 2017; Accepted: May 7, 2018
Proofs received from author(s): July 15, 2018