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Partial validation of a TaqMan real-time quantitative PCR for the detection of ranaviruses

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ABSTRACT: Ranaviruses are globally emerging pathogens negatively impacting wild and cultured fish, amphibians, and reptiles. Although conventional and diagnostic real-time PCR (qPCR) assays have been developed to detect ranaviruses, these assays often have not been tested against the known diversity of ranaviruses. Here we report the development and partial validation of a TaqMan real-time qPCR assay. The primers and TaqMan probe targeted a conserved region of the major capsid protein (MCP) gene. A series of experiments using a 10-fold dilution series of *Frog virus 3* (FV3) MCP plasmid DNA revealed linearity over a range of 7 orders of magnitude (10^7–10^1), a mean correlation coefficient (R^2) of >0.99, and a mean efficiency of 96%. The coefficient of variation of intra- and inter-assay variability ranged from <0.1–3.5% and from 1.1–2.3%, respectively. The analytical sensitivity was determined to be 10 plasmid copies of FV3 DNA. The qPCR assay detected a panel of 33 different ranaviral isolates originating from fish, amphibian, and reptile hosts from all continents excluding Africa and Antarctica, thereby representing the global diversity of ranaviruses. The assay did not amplify highly divergent ranaviruses, members of other iridovirus genera, or members of the alloherpesvirus genus *Cyprinivirus*. DNA from fish tissue homogenates previously determined to be positive or negative for the ranavirus *Epizootic hematopoietic necrosis virus* by virus isolation demonstrated a diagnostic sensitivity of 95% and a diagnostic specificity of 100%. The reported qPCR assay provides an improved expedient diagnostic tool and can be used to elucidate important aspects of ranaviral pathogenesis and epidemiology in clinically and sublinically affected fish, amphibians, and reptiles.

KEY WORDS: Ranavirus · Quantitative PCR · Diagnostics · Sensitivity · Specificity

INTRODUCTION

Since the isolation of *Frog virus 3* from a northern leopard frog *Lithobates pipiens* in the late 1960s, detections of ranaviruses have expanded to include a range of species across 3 classes of ectothermic vertebrates (Osteichthyes, Amphibia, Reptilia) inhabiting temperate and tropical environments worldwide (Duffus et al. 2015). The impact of ranaviruses on cultured and wild populations of endangered ectother-

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mic vertebrates is recognized as an important conservation concern (Geng et al. 2011, Miller et al. 2011, Waltzek et al. 2014, Cunningham et al. 2016). As a result, ranaviral disease in amphibians and disease due to the fish ranavirus *Epizootic hematopoietic necrosis virus* (EHNV) are notifiable to the World Organisation for Animal Health (OIE 2016).

Members of the family *Iridoviridae* include large double-stranded DNA viruses with polygonal nucleocapsids observed within cytoplasmic assembly sites (Chinchar et al. 2017). The family has recently been reorganized into the subfamily *Alphairidovirinae* (genera *Ranavirus*, *Lymphocystivirus*, and *Megalocytivirus*) that infect ectothermic vertebrates and subfamily *Betairidovirinae* (genera *Iridovirus* and *Chloroiridovirus*) that infect invertebrates such as insects and crustaceans (Chinchar et al. 2017). Seven ranaviral species are recognized by the International Committee on Taxonomy of Viruses (ICTV): *Ambystoma tigrinum virus* (ATV), *Bohle iridovirus* (BIV), EHNV, *European catfish virus* (ECV), *Frog virus 3* (FV3), *Santee-Cooper ranavirus* (SCRV), and the recently added *Singapore grouper iridovirus* (SGIV) (Chinchar et al. 2017). Increased awareness and surveillance for ranaviruses as well as the advent of high-throughput sequencing technologies have resulted in the discovery and genetic characterization of a large number of ranaviruses yet to be classified by the ICTV.

Phylogenomic comparison of ranaviruses isolated from cultured Chinese reptiles and amphibians including the tiger frog virus (TFV; He et al. 2002), soft-shelled turtle iridovirus (STIV; Huang et al. 2009) and *Rana grylio* virus (RGV; Lei et al. 2012) has revealed that they belong to a novel clade that includes FV3 and BIV and are collectively referred to as the FV3/BIV/TFV-like viruses (Jancovich et al. 2015). A more recently described clade of ranaviruses has caused epidemics in highly endangered giant salamanders in Chinese rearing facilities (*Andrias davidianus* ranavirus, ADRV; Chen et al. 2013) as well as European amphibians including the common midwife toad *Alytes obstetricans* (common midwife toad virus, CMTV; Price et al. 2014). Sequencing of the pike-perch iridovirus (PPIV) genome, isolated in Finland from apparently healthy pike-perch *Sander lucioperca* fingerlings in 1995, revealed PPIV as the first member of the CMTV/ADRV-like ranaviral clade to be isolated from fish (Holopainen et al. 2016). Finally, phylogenomic analyses of the short-finned eel ranavirus (SERV; Subramaniam et al. 2016b), cod iridovirus (CoIV), and Ranavirus maxima (Rmax) isolated from turbot revealed that these fish ranaviruses branch off at the base of the ranaviral tree near other fish ranaviruses, with only the highly divergent SCRV and grouper iridoviruses splitting off earlier (Ariel et al. 2016).

Although clinical presentation varies among host species, ranaviruses generally induce systemic disease manifesting as external and internal hemorrhages, edema (particularly in amphibians and chelonians), behavioral changes (e.g. lethargy, anorexia, erratic movement), and significant morbidity/mortality (Miller et al. 2015, Rijks et al. 2016). According to the OIE diagnostic manual for ranaviral infection in amphibians (OIE 2016), a case is suspect for ranaviral infection if the skin and/or parenchymal tissues of an apparently healthy, moribund, or dead individual contain histological evidence of necrosis with or without the presence of intracytoplasmic basophilic inclusion bodies. A case is confirmed when the suspect animal's tissues or cell culture test positive via (1) immunoperoxidase test/stain, (2) antigen-capture ELISA, (3) PCR followed by restriction enzyme analysis (REA) or sequencing, and/or (4) immunoelectron microscopy (tissue only). For EHNV infections in fish (OIE 2016), a suspect case is defined when 1 or more animals demonstrates characteristic histopathology (e.g. liquefactive or coagulative necrosis) with or without the presence of intracytoplasmic inclusion bodies. Suspect EHNV cases are confirmed via PCR (Hyatt et al. 2000) with sequencing or REA, plus 1 or more of the following: (1) immunoperoxidase test/stain (Reddacliff & Whittington 1996), (2) antigen-capture ELISA, or (3) immunoelectron microscopy.

End-point PCR (conventional) and real-time quantitative PCR (qPCR) assays have been developed for the detection of ranaviruses (Table 1). Most target the major capsid protein (MCP) gene (Mao et al. 1997, Hyatt et al. 2000, Marsh et al. 2002, Pallister et al. 2007). The MCP gene is ideal for molecular diagnostic assays because it is highly conserved and maintains stable base mutations that allow for the differentiation of species (Mao et al. 1997). Two conventional PCR assays targeting the MCP are recommended by the OIE (2016) for diagnosis of ranaviruses. One assay allows for the differentiation of Australian ranaviral species (EHNV and BIV) from American (FV3) and European (ECV) species by performing REA of PCR amplicons (Marsh et al. 2002). The second conventional assay targets a 580 bp region of the MCP which can be sequenced for ranaviral species identification (Hyatt et al. 2000). Real-time qPCR assays have been designed to detect specific ranaviruses (Goldberg et al. 2003, Getchell et al. 2007, Allender et al. 2013) or a range of ranaviruses (Pallister et al. 2007, Holopainen et al.

Table 1. Quantitative PCR assays developed for the detection of ranaviruses. See Table 3 for taxa abbreviations

Assay	Purpose	Gene target	Method	Virus(es) evaluated	Validation for diagnostic use	Reference
1	Quantification	MCP	TaqMan qPCR	LMBV	No	Goldberg et al. (2003)
2	Quantification	MCP	TaqMan qPCR	LMBV	Yes	Getchell et al. (2007)
3	Detection and differentiation	MCP	TaqMan qPCR	BIV, ECV, ESV, EHNV	No	Pallister et al. (2007)
4	Quantification	DNA polymerase	SYBR Green qPCR	EHNV, ECV, ESV, FV3, BIV, DFV, GV6, PPIV, REV, SERV	No	Holopainen et al. (2011)
5	Detection and differentiation	MCP	SYBR Green qPCR	BIV, Cum5, Cum6, Cum30, EHNV, ECV, ESV, FV3, GV, Mat2, Mg1, MHRV, TEV, UKIV1, WV	Yes	Jaramillo et al. (2012)
6	Detection and quantification	MCP	TaqMan qPCR	FV3	Partial (sensitivity and specificity)	Allender et al. (2013)

2011, Jaramillo et al. 2012) (Table 1). Of these assays, only 1 SYBR Green qPCR assay has been evaluated against a number of ranaviral taxa (Jaramillo et al. 2012).

The documented increase in ranaviral epidemics negatively impacting ectothermic vertebrate populations globally, the dissemination of previously geographically restricted ranaviruses (e.g. Wamena virus) through the unregulated international trade in exotic animals (Hyatt et al. 2002), and the recent characterization of novel ranaviral lineages all underscore the need for diagnostic tools capable of detecting genetically diverse ranaviruses infecting a range of hosts from disparate regions of the world (Whittington et al. 2010, Duffus et al. 2015). Currently available ranavirus-specific assays have not been validated for the full diversity of ranaviruses; thus, we developed and partially validated a TaqMan real-time qPCR assay capable of detecting most genetic lineages of ranaviruses.

MATERIALS AND METHODS

The following steps to develop and partially validate a ranavirus TaqMan real-time qPCR assay were carried out at the University of Florida's Wildlife and Aquatic Animal Veterinary Disease Laboratory (WAVDL) in Gainesville, FL, USA: (1) *in silico* primer and probe design, (2) estimation of the analytical sensitivity and specificity, slope, *y*-intercept, correlation coefficient (R^2), efficiency, and dynamic range, and (3) estimation of the repeatability and reproducibility as determined from 12 experiments (plates). Diagnostic sensitivity and specificity were evaluated at the OIE Reference Laboratory (OIERL) for EHNV and amphibian ranaviruses at the University of Sydney in Camden, Australia. The University of Sydney node of the OIERL also tested a subset of ranaviral isolates as part of determining the analytical specificity of the qPCR assay.

In silico TaqMan qPCR primer and probe design

Sequences for the 26 iridovirus core genes (Eaton et al. 2007) were obtained from GenBank for 18 fully sequenced ranaviruses. Each gene was aligned in MAFFT (Katoh & Toh 2008) using default settings. Examination of the initial alignments revealed the Santee-Cooper ranaviruses (doctor fish virus, DFV; guppy virus 6, GV6; largemouth bass virus, LMBV) and grouper iridoviruses (grouper iridovirus, GIV; *Singapore grouper iridovirus*, SGIV) are highly divergent from other ranaviruses and thus, they were excluded from all gene alignments. The alignments were imported into Geneious 7.0 to generate a consensus sequence with the threshold set to 100% (Kearse et al. 2012). The consensus sequences for each gene were then imported into PrimerExpress v2.0 (Applied Biosystems) to design pan-ranaviral primers and hydrolysis probes using default settings. The consensus sequence of the major capsid protein returned the only suitable primers (RanaF1 and RanaR1) and probe (RanaP1) combination (Table 2). The homologous MCP sequences were then obtained for an additional 18 partially sequenced ranaviruses and the resulting 36 sequences were then aligned and visualized in Bioedit v7.2.5 using default settings (Fig. 1, Table 3).

Table 2. Primers and probes designed against the ranavirus major capsid protein (MCP) gene for use in the diagnostic assay and development of the frog virus 3 (FV3) plasmid standard. MCP gene positions 891 and 1249 correlate to positions 98235 and 98593 in the FV3 genome (GenBank accession no. AY548484)

Primer/probe name	Primer/probe sequence	Melting temp. (°C)	Positions in MCP gene (5′ to 3′)	Amplicon size (nt) including primers
RanaMCPstdF	GTT CTC ACA CGC AGT CAA GG	53.8	891–910	359
RanaMCPstdR	CGG ACA GGG TGA CGT TAA G	53.2	1231–1249	
RanaF1	CCA GCC TGG TGT ACG AAA ACA	54.4	1040–1060	97
RanaR1	ACT GGG ATG GAG GTG GCA TA	53.8	1136–1117	
RanaP1	6FAM-TGG GAG TCG AGT ACT AC-MGB	47.1	1079–1095	

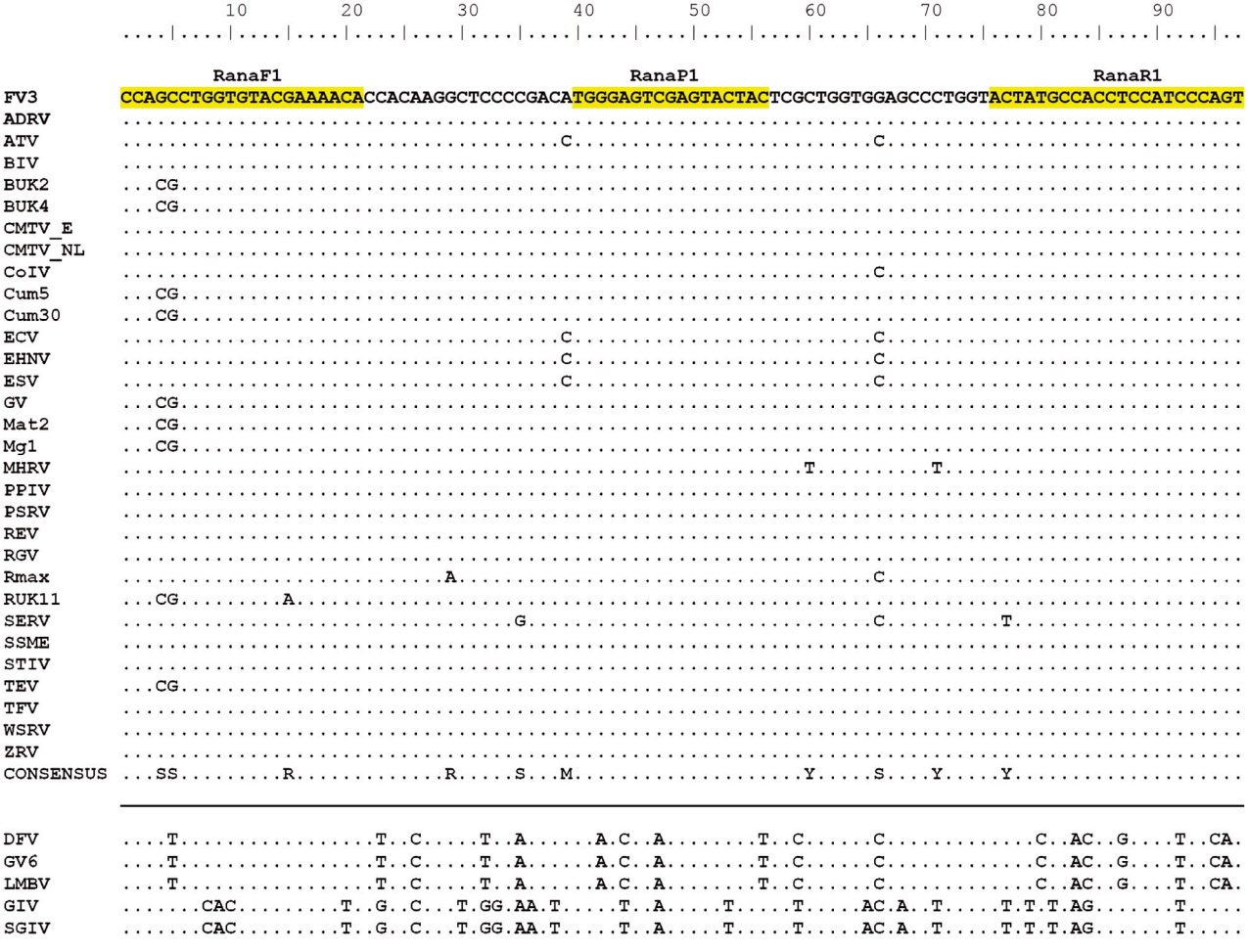

Fig. 1. Aligned partial (97 bp) major capsid protein (MCP) sequences for 36 ranaviruses illustrating the *in silico* specificity of the qPCR primers (RanaF1 and RanaR1) and TaqMan probe (RanaP1). Divergent ranaviruses are shown below the solid black line (see Table 3 for viral abbreviations and GenBank accessions)

Detection of ranaviral DNA using the qPCR assay

At WAVDL, the qPCR assay reactions were carried out in 20 µl volumes containing 0.9 µM of each primer, 0.25 µM of probe, 4 µl of nucleic acid template, 10 µl of universal qPCR mix (TaqMan® Fast Universal PCR Master Mix 2×, Applied Biosystems), and 3 µl of molecular-grade water. Reactions were run on a 7500 Fast Real-Time PCR System (Applied Biosystems) using the standard Fast protocol thermocycling conditions: 95°C for 20 s, followed by 45 cycles at 95°C for 3 s and

Table 3. Panel of ranaviruses used for primer and probe design and/or validation of the TaqMan qPCR assay. Positive (+) and negative (−) symbols indicate expected specificity with the primer/probe design or the actual qPCR TaqMan test result. Viral species names accepted by the International Committee on Taxonomy of Viruses are italicized. NE indicates the sample was not evaluated in this study

Virus species/isolate	Abbreviation	Geographic origin	GenBank accession	Primer/ probe design	qPCR result	Reference
Andrias davidianus ranavirus	ADRV	China	KC865735	+	NE	Chen et al. (2013)
Ambystoma tigrinum virus	ATV	Arizona	AY150217	+	+	Jancovich et al. (2003)
Bohle iridovirus	BIV	Australia	KX185156	+	+	Hick et al. (2016)
Bufo bufo United Kingdom iridovirus 1[a]	BUK2	UK	AF157653	+	+	Hyatt et al. (2000)
Bufo bufo United Kingdom iridovirus 3[a]	BUK4	UK	AF157657	+	+	Hyatt et al. (2000)
Bufo marinus Venezuelan iridovirus 1[a]	GVB3	Venezuela	AF157649	+	+	Hyatt et al. (2000)
Bufo marinus Venezuelan iridovirus 2[a]	Mg1	Venezuela	AF157677	+	+	Hyatt et al. (2000)
Bufo marinus Venezuelan iridovirus 3[a]	Mat2	Venezuela	AF157675	+	+	Hyatt et al. (2000)
Bufo marinus Venezuelan iridovirus 4[a]	Cum5	Venezuela	AF157663	+	+	Hyatt et al. (2000)
Bufo marinus Venezuelan iridovirus 5[a]	Cum6	Venezuela		NE	+	Hyatt et al. (2000)
Bufo marinus Venezuelan iridovirus 6[a]	Cum30	Venezuela	AF157661	+	+	Hyatt et al. (2000)
Epizootic hematopoietic necrosis virus	EHNV	Australia	FJ433873	+	+	Jancovich et al. (2010)
Common midwife toad virus	CMTV_E	Spain	JQ231222	+	NE	Mavian et al. (2012a)
Common midwife toad virus	CMTV_NL	Netherlands	KP056312	+	+	van Beurden et al. (2014)
European sheatfish virus	ESV	Germany	JQ724856	+	+	Mavian et al. (2012b)
Fathead minnow ranavirus	FHMRV	Arkansas		NE	+	Waltzek et al. (2014)
Frog virus 3	FV3	Wisconsin/ Minnesota	AY548484	+	+	Tan et al. (2004)
Grouper iridovirus[b]	GIV	Taiwan	AY666015	−	NE	Tsai et al. (2005)
Guppy virus 6[b]	GV6	USA (imported from Asia)	FR677325	−	−	Ohlemeyer et al. (2011)
Infectious spleen and kidney necrosis virus[b]	ISKNV	USA		NE	−	T. B. Waltzek (unpubl.)
Largemouth bass virus[b]	LMBV	Mississippi	FR682503	−	−	Ohlemeyer et al. (2011)
Lymphocystis disease virus[b]	LCDV	USA		NE	−	T. B. Waltzek (unpubl.)
Mahaffey Road virus	MHRV	Australia	GU292010	+	+	Weir et al. (2012)
Marbled sleepy goby virus	MSGV	Thailand		NE	+	Prasankok et al. (2005)
Northern pike ranavirus	NPRV	Ohio		NE	+	Waltzek et al. (2014)
Pike-perch iridovirus	PPIV	Finland	KX574341	+	+	Holopainen et al. (2016)
Pallid sturgeon ranavirus	PSRV	Missouri	KF646249	+	+	Waltzek et al. (2014)
Rana esculenta virus	REV	Italy	FJ358611	+	+	Holopainen et al. (2009)
Rana grylio virus	RGV	China	JQ654586	+	NE	Lei et al. (2012)
Rana temporaria United Kingdom iridovirus 1[a]	RUK11	UK	AF157645	+	+	Hyatt et al. (2000)
Russian sturgeon ranavirus	RSRV	Georgia		NE	+	Waltzek et al. (2014)
Short-finned eel ranavirus	SERV	Italy	NC_030394	+	+	Subramaniam et al. (2016b)
Singapore grouper iridovirus[b]	SGIV	Singapore	NC_006549	−	NE	Song et al. (2004)
Softshell turtle iridovirus	STIV	China	EU627010	+	NE	Huang et al. (2009)
Spotted salamander Maine virus	SSME	Maine	KJ175144	+	+	Morrison et al. (2014)
Tadpole edema virus[a]	TEV	North America	AF157681	+	+	Hyatt et al. (2000)
Tiger frog virus	TFV	China	AF389451	+	NE	He et al. (2002)
Wamena virus[a]	WV	Australia (imported from Indonesia)		NE	+	Hyatt et al. (2002)
White sturgeon ranavirus	WSRV	California		+	+	Waltzek et al. (2014)
Zoo ranavirus	ZRV	Iowa	KF699143	+	+	Cheng et al. (2014)

[a]Sample was tested by the OIE Reference Laboratory for EHNV and amphibian ranaviruses
[b]Divergent viruses not expected to amplify with the ranavirus TaqMan qPCR primers and probe

60°C for 30 s. A cycle threshold (Ct) was calculated and interpreted as a positive result for samples if the 6-carboxy-X-rhodamine (ROX)-normalized 6-carboxyfluorescein (FAM) signal exceeded the threshold assigned by the Applied Biosystems software.

Polypropylene plates (Olympus Plastics, Genesee Scientific) sealed with 50 µm polyolefin films (ThermalSeal RTS, Excel Scientific) included at least 2 no-template negative controls (molecular grade water). Each sample was run in triplicate with the ranavirus-specific primers and probe and singly with an 18S

rRNA exogeneous control assay (Applied Biosystems Assay ID Hs99999901_s1) with a final reaction concentration of 0.9 µM of each primer and 0.25 µM of probe. All experiments involved the probe with a minor-groove binding quencher to increase specificity, reduce background fluorescence, and increase the signal-to-noise ratio. A 10-fold dilution series of linearized plasmid DNA (10^7–10^1 copies), carrying a 359 bp fragment of the FV3 MCP gene (Table 2) amplified from an isolate recovered from pallid sturgeon *Scaphirhynchus albus* during a 2015 hatchery epidemic (N. K. Stilwell & T. B. Waltzek unpubl.), was amplified in triplicate on each plate. Efficiency was calculated as $10^{-1/slope} - 1$ (Bustin et al. 2009).

The qPCR assay was also evaluated in the OIERL. Samples were tested in duplicate 20 µl reactions using Path-ID qPCR Master Mix (ThermoFisher Scientific), 0.9 µM of each primer, 0.25 µM probe, and 4 µl of nucleic acid template. Thermocycling was performed on an Mx3000 qPCR system (Strategene) with the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 30 s and 60°C for 45 s. Ct was calculated and interpreted as a positive result for samples if the ROX-normalized FAM signal exceeded the threshold assigned by the MxPro software (Stratagene).

Estimation of qPCR assay parameters

Triplicate 10-fold dilutions of the FV3 plasmid DNA standard were used in each of the 12 experiments (plates) run at WAVDL to estimate the slope, *y*-intercept, correlation coefficient (R^2), efficiency, dynamic range, analytical sensitivity, repeatability, and reproducibility of the qPCR assay. The qPCR assay limit of detection (LOD or analytical sensitivity) was defined as the lowest dilution at which 50% of positive samples (wells) were detected (OIE 2016). The percent coefficient of variation (CV% = [SD/mean] × 100%) for intra-assay (repeatability) and inter-assay (reproducibility) variability were calculated as the mean and SD of the Ct values within (repeatability) or among (reproducibility) the 12 experiments.

To estimate the qPCR assay analytical specificity, 36 reference ranavirus isolates were grown either at WAVDL (25) or the OIERL (11) (Table 3). Ranaviruses were amplified in either epithelioma papulosum cyprini (EPC) or bluegill fry (BF-2) cell lines maintained in minimal essential medium with 10% fetal bovine serum at 23°C until complete destruction of the cell monolayer was observed. DNA was extracted from tissue culture supernatants using a QIAcube with the DNeasy Blood and Tissue Extraction protocol (Qiagen). Extracted DNA was quantified using a Qubit® 3.0 Fluorometer (LifeTechnologies). DNA from the 36 reference isolates was used to determine the detectability of each isolate. The qPCR assay was also tested at WAVDL against DNA from tissues infected with related iridoviruses in the genera *Lymphocystivirus* (LCDV) and *Megalocytivirus* (ISKNV) and 3 alloherpesviruses in the genus *Cyprinivirus* (CyHV1-3) (Table 3). The LCDV-infected tissue DNA was extracted from the fin of a ram cichlid *Mikrogeophagus ramirezi* displaying the stereotypical gross proliferative lesions typical for the disease and was confirmed by PCR and Sanger sequencing of the partial viral DNA polymerase gene as previously described (Hanson et al. 2006). The ISKNV and CyHV1-3 infected tissue DNA samples have been described by Subramaniam et al. (2016a) and Viadanna et al. (2017), respectively.

Estimation of qPCR assay diagnostic sensitivity and specificity

DNA was extracted and purified from tissue homogenates of pooled kidney-liver-spleen from EHNV-exposed redfin perch *Perca fluviatilis* (Becker et al. 2016), Murray–Darling rainbowfish *Melanotaenia fluviatilis*, eastern mosquitofish *Gambusia holbrooki*, freshwater catfish *Tandanus tandanus*, Macquarie perch *Macquaria australasica*, and silver perch *Bidyanus bidyanus* including both clinically and subclinically affected individuals as described previously (Becker et al. 2013). Corresponding EHNV-free control fish were also evaluated (Becker et al. 2013) (Table 4). Briefly, samples were determined to be positive or negative for EHNV based on isolation of the virus in BF-2 cells. Cytopathic effect (CPE) was confirmed to be due to EHNV by PCR (Marsh et al. 2002) or qPCR (Jaramillo et al. 2012) on infected tissue culture supernatant. Samples were determined to be negative for EHNV if virus isolation was negative after 3 passes in cell culture. The purified nucleic acid samples from tissue homogenates had been archived at −80°C prior to this study. In total, 106 virus isolation positive fish samples representing individuals with both clinical and subclinical infection and 80 virus isolation negative fish tissue samples were used to estimate the diagnostic sensitivity and specificity of the qPCR assay (Table 4).

Table 4. Estimation of the TaqMan qPCR assay diagnostic sensitivity and specificity performed at the OIE Laboratory for the fish ranavirus *Epizootic hematopoietic necrosis virus* (EHNV)

Expected EHNV status	Fish species	Virus isolation	qPCR assay Positive (n)	qPCR assay Negative (n)
Negative (–)	Redfin perch *Perca fluviatilis*	–	0	36
	Freshwater catfish *Tandanus tandanus*	–	0	4
	Trout cod *Maccullochella macquariensis*	–	0	40
	Total, all species		0	80
Positive (+)	Redfin perch	+	70	1
	Freshwater catfish	+	2	0
	Mosquito fish *Gambusia holbrooki*	+	23	1
	Silver perch *Bidyanus bidyanus*	+	0	3
	Murray-Darling rainbow fish *Melanotaenia fluviatilis*	+	5	0
	Macquarie perch *Macquaria australasica*	+	1	0
	Total, all species		101	5

RESULTS

In silico TaqMan qPCR primer and probe design

Our initial examination of the *in silico* specificity of the RanaF1 primer revealed 2 or 3 potential mismatches in 9 ranaviral isolates (Cum5, Cum30, BUK2, BUK4, GVB3, Mat2, Mg1, RUK11, TEV) originally sequenced by Hyatt et al. (2000). The partial MCP sequences of these isolates (Table 3) all display a CG at nucleotide positions 4 and 5 (Fig. 1).

However, Marsh et al. (2002) later sequenced the full length MCP gene sequence for a subset of the same isolates (BIV, EHNV, FV3) and corrected the aforementioned CG to a GC. Furthermore, complete genome sequencing of some of the same isolates (FV3, EHNV, BIV, RUK11, TEV) support the GC as observed in every other ranavirus displayed in Fig. 1 with the exception of the Hyatt et al. (2000) sequences and sequences from the divergent ranaviruses DFV, GV6, and LMBV that display a GT at positions 4 and 5 (Tan et al. 2004, Jancovich et al. 2010, Hick et al. 2016, T. B. Waltzek unpubl.). In addition to the aforementioned potential mismatches in positions 4 and 5, RUK11 and RUK13 both possess a third primer mismatch at position 15 encoding an adenine (Hyatt et al. 2000). However, sequencing of the RUK13 genome (GenBank accession no. KJ538546) does not support the previously reported Sanger sequence that would result in RanaF1 primer mismatches in positions 4, 5, and 15. To date, none of the potential mismatches in the RanaF1 primer have been confirmed, and sequencing of >70 ranavirus genomes at WAVDL has yet to produce a mismatch in the herein described primers or probe (Table 2, T. B. Waltzek unpubl. data).

Estimation of qPCR assay parameters

The amplification plot revealed that the qPCR assay was linear over 7 orders of magnitude (10^7–10^1) (Fig. 2). Amplification of the 10^0 standard was inconsistent, and a 10^8 standard sample produced an abnormally shaped standard curve, presumably due to high DNA concentration. The mean parameters (±SE) for the qPCR assay averaged over the 12 experiments (plates) were as follows: slope = −3.42 ± 0.02, *y*-intercept = 40.57 ± 0.26, R^2 = 0.998 ± 0.0006, and efficiency = 96.82 ± 0.55% (Fig. 2B). The LOD of the assay (analytical sensitivity) was determined to be 10 plasmid copies of FV3 DNA (positive in 93% of the reactions). The coefficient of variation of intra- and inter-assay mean Ct values ranged from <0.1–3.5% and 1.1–2.3%, respectively (Table 5).

The analytical specificity confirmed the *in silico* design of the assay (Fig. 1), with ranaviral DNA amplifying 33 ranaviral DNA samples belonging to 5/6 tested ranaviral species as well as taxonomically unclassified ranaviruses (Table 3). The 3 tested divergent ranaviruses (DFV, GV6, LMBV), iridoviruses from other genera (i.e. ISKNV in the genus *Megalocytivirus* and LCDV in the genus *Lymphocystivirus*), and alloherpesviruses in the genus *Cyprinivirus* (CyHV1-3) were all negative as expected from the *in silico* design of the assay. The 18s rRNA internal controls were positive for all samples, indicating that the samples contained sufficient DNA for amplification.

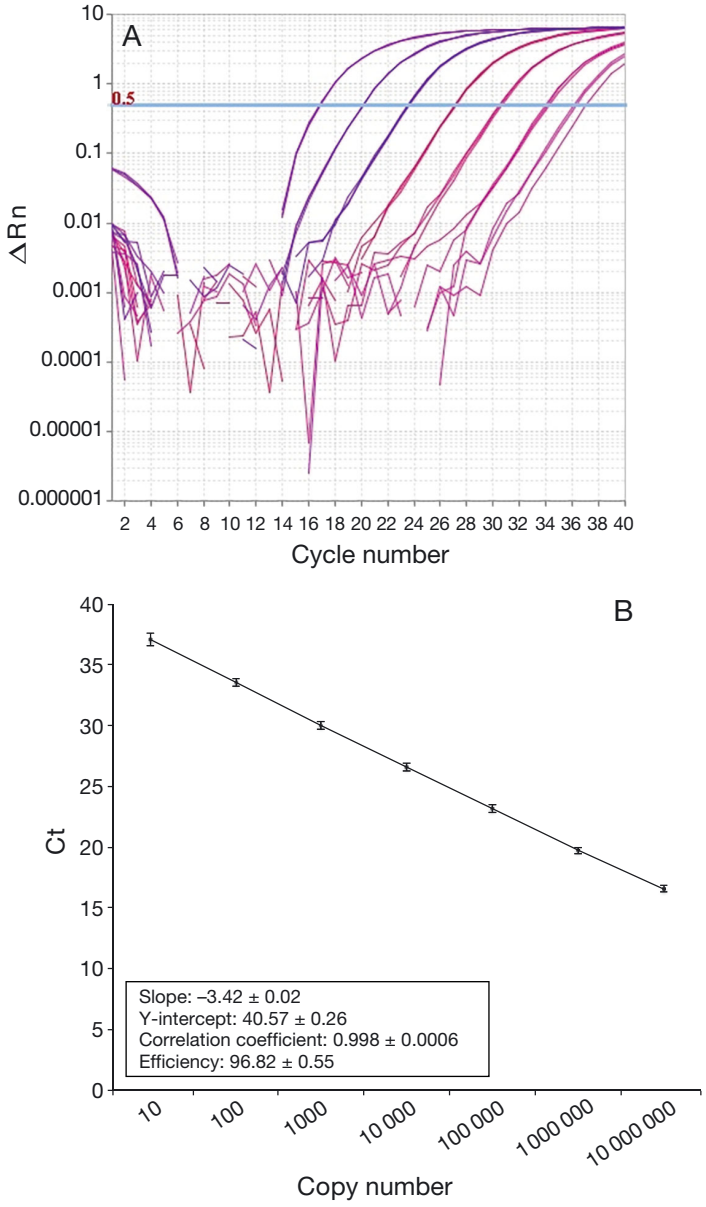

Fig. 2. Ranavirus TaqMan qPCR assay (A) amplification plot and (B) standard curve generated using triplicate 10-fold serial dilutions of the frog virus 3 plasmid DNA standards ranging from 10^7–10^1 copies. In (A), a typical experiment is shown with the blue line indicating the automatic threshold assigned by the Applied Biosystems software. The *x*-axis indicates the cycle number and the *y*-axis indicates the ΔRn. The normalized reporter (Rn) signal is the fluorescence emission intensity of the reporter dye (FAM) divided by the fluorescence emission intensity of the passive reference dye (ROX). The ΔRn is the magnitude of the signal generated during the PCR at each time point as determined by the following equation: ΔRn = (Rn+) – (Rn). Rn+ is the Rn value of a reaction containing all components, including the template and Rn is the Rn value of an unreacted sample. In (B), the mean qPCR assay parameters (±SE) averaged over the 12 experiments (plates) are provided. The *x*-axis shows the plasmid standard copy number and the *y*-axis indicates the corresponding cycle threshold (Ct) value

Estimation of qPCR assay diagnostic sensitivity and specificity

Testing of 106 virus isolation positive EHNV-infected samples and 80 virus isolation negative samples indicated a diagnostic sensitivity of 95% (95% confidence limits: 89.3–98.5%) and diagnostic specificity of 100% (95.5–100%) when compared to viral isolation as a standard test (Table 4).

DISCUSSION

The availability of a validated real-time qPCR assay capable of detecting all members of the genus *Ranavirus* has become an increasingly important goal given the expansion of the geographic and host ranges of known ranaviruses (Duffus et al. 2015). The qPCR assay reported here was optimized and partially validated to detect all known ranaviruses excluding the most divergent species (SCRV and SGIV) and members of the other iridovirus genera *Lymphocystivirus* and *Megalocytivirus*. SCRV and SGIV were deliberately excluded during our initial assay design as their inclusion in the genus has been questioned (Whittington et al. 2010, Jancovich et al. 2015) and because specific SCRV qPCR assays have previously been developed (Goldberg et al. 2003, Getchell et al. 2007). The qPCR assay detected 33 ranaviruses including 5 of 6 recognized species (ATV, BIV, ECV, EHNV, FV3) as well as unclassified ranaviruses from fish, amphibians, and reptiles inhabiting varied ecosystems in North and South America, Europe, Asia, and Australia (Table 3). Several species and/or genetic clades were represented by more than 1 isolate in testing (e.g. FHMRV, NPRV, RSRV, PSRV, and SSME isolates have been confirmed via genomic

Table 5. Inter-assay variability (reproducibility) of the pan-ranavirus qPCR across 12 experiments (plates) at the University of Florida's Wildlife and Aquatic Animal Veterinary Disease Laboratory. Reactions for each plasmid standard (10^7–10^1 copies) were run in triplicate. Ct: cycle threshold

Standard dilution	Ct Mean	Ct SD	CV (%)	No. of wells positive (/36)
10^7	16.57	0.27	1.6	36
10^6	19.73	0.27	1.4	36
10^5	23.18	0.31	1.4	36
10^4	26.57	0.35	1.3	36
10^3	30.04	0.34	1.1	36
10^2	33.58	0.37	1.1	36
10^1	37.09	0.53	2.3	33

sequencing as strains of FV3) (N. K. Stilwell et al. unpubl.).

Although a number of qPCR assays to detect ranaviruses have been developed (Table 1), some were designed to specific ranaviruses and none have been fully validated for diagnostic application with the exception of a SYBR Green qPCR assay designed by Jaramillo et al. (2012). Their assay was partially validated for diagnostic application and detected all 20 ranaviruses tested. While a few potential mismatches were noted in the RanaF1 primer of our TaqMan real-time qPCR assay (Fig. 1), a greater number of mismatches were noted when mapping the SYBR Green qPCR assay primers of Jaramillo et al. (2012) onto the ranaviral MCP sequences assessed in this study (Fig. A1 in the Appendix). Primer mismatches could negatively impact assay sensitivity (Green & Sambrook 2012). On balance, our study and that of Jaramillo et al. (2012) reported high assay efficiency, analytical/diagnostic sensitivity and specificity, and repeatability. Thus, the partially validated TaqMan qPCR assay presented here and the aforementioned SYBR Green qPCR assay both lend themselves to diagnostic applications including confirmation of clinical cases and surveillance.

The qPCR assay reported here detected a wide range of ranaviruses from different hosts and continents; however, the assay cannot discriminate between ranaviral species or strains. Species discrimination can be achieved using OIE recommended conventional PCR assays targeting the MCP gene (Hyatt et al. 2000, Marsh et al. 2002). More recently, next-generation sequencing technologies to sequence ranaviral genomes can now facilitate phylogenomic analyses to discriminate ranaviral species or strains (Ariel et al. 2016, Hick et al. 2016, Holopainen et al. 2016, Subramaniam et al. 2016b).

In this report, we completed stages 1 (analytical characteristics) and 2 (diagnostic characteristics) of the OIE criteria for assay development and validation (OIE 2016). Experiments using plasmid standards demonstrated high assay efficiency, correlation coefficient, repeatability, reproducibility, and analytical sensitivity and specificity. The LOD of the assay was 10 copies of FV3 plasmid DNA (Table 5). The assay performed well against a panel of known EHNV positive and negative fish tissue samples, indicating its high diagnostic sensitivity (95%) and specificity (100%) (Table 4). The 5 positive fish tissue samples that tested negative (false negatives) were all samples with low EHNV viral loads that may have been below the assay LOD. Reduced sample quality following long-term storage or PCR inhibition are possible alternative explanations for these false negative results (Jaramillo et al. 2012). The performance of the assay for detecting ranavirus DNA in amphibian and chelonian tissues has not been fully evaluated (exceptions discussed below). However, we expect similar performance to that in fish due to similar pathologies and tissue constitution in these species, as well as high degrees of virus homology, all suggesting ease of release of virus and purification and detection of viral DNA.

In partial fulfillment of stages 3 (reproducibility) and 4 (implementation) in the OIE criteria for assay development and validation, the qPCR assay has routinely been used by WAVDL for the last 2 yr, providing insights into the pathogenesis of FV3 in pallid sturgeon *Scaphirhynchus albus* following experimental challenges (N. K. Stilwell et al. unpubl.) and FV3 epidemics within cultured and wild herpetofauna. The University of Sydney node of the OIERL has implemented the assay in surveillance of cane toad populations in Australia for approximately 1 yr, and more recently, in other US university laboratories that routinely conduct ranaviral diagnostics.

The reported qPCR assay offers an expedient and cost-effective test for the diagnosis of ranaviruses in both tissue culture supernatants and tissues, making this tool particularly useful for understanding ranaviral pathogenesis and epidemiology. As this assay was created and validated using a diverse group of fish, amphibian, and reptile ranaviruses from around the world, it serves as an important tool in ranaviral surveillance programs aimed at the management and mitigation of these globally emerging pathogens.

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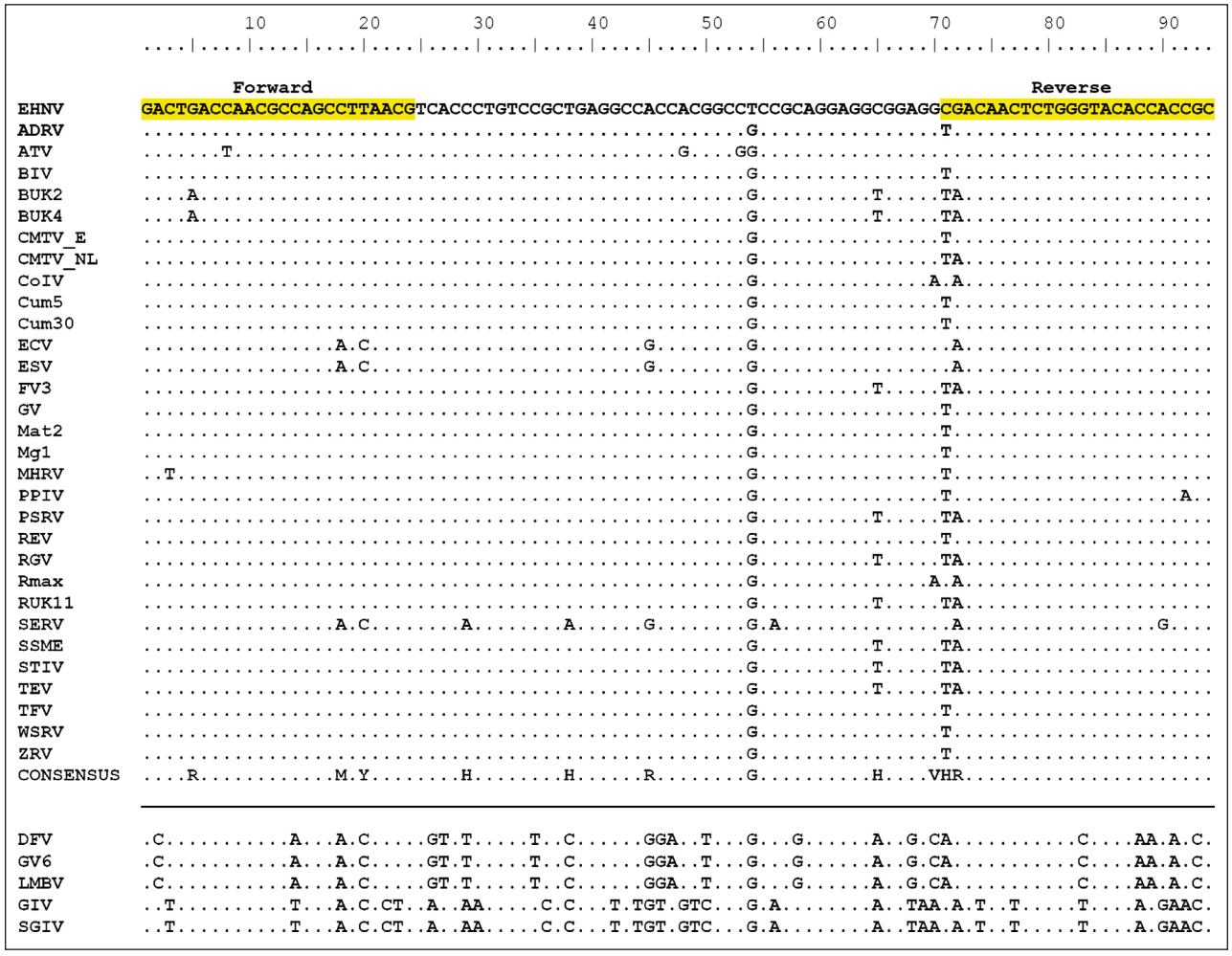
Appendix

Fig. A1. Aligned partial (94 bp) major capsid protein (MCP) sequences for 36 ranaviruses illustrating *in silico* specificity of the SYBR green qPCR primers developed by Jaramillo et al. (2012). Divergent ranaviruses are shown below the solid black line (see Table 3 for viral abbreviations and GenBank accession numbers)



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