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Development and validation of TaqMan quantitative PCR for detection of frog virus 3-like virus in eastern box turtles (*Terrapene carolina carolina*)

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A B S T R A C T

Ranavirus has caused disease epidemics and mass mortality events globally in free-ranging fish, amphibian, and reptile populations. Viral isolation and conventional PCR are the most common methods for diagnosis. In this study, a quantitative real-time PCR (qPCR) assay was developed using a TaqMan probe-based assay targeting a highly conserved region of the major capsid protein of frog virus 3-like virus (FV3-like) (Family *Iridoviridae*, genera *Ranavirus*). Standard curves were generated from a viral DNA segment cloned within a plasmid. The assay detected viral DNA 1000 times lower than conventional PCR. Thirty-one clinical samples (whole blood and oral swabs) from box turtles were tested using these assays and the prevalence of the virus determined. Quantitative PCR allows for a superior, rapid, sensitive, and quantitative method for detecting FV3-like virus in box turtles, and this assay will be useful for early detection and disease monitoring.

1. Introduction

Ranavirus is one of five genera from the family *Iridoviridae* and one of three genera reported to infect reptiles. They are large, icosohedral, DNA viruses that have emerged as a significant cause of mortality events in free-ranging herpetofauna throughout the world (Green et al., 2002; Johnson et al., 2008; Miller et al., 2011). *Ranavirus* has specifically been linked as a contributing factor in amphibian declines, with numerous outbreaks occurring in the US (Green et al., 2002; Jancovich et al., 2003; Johnson et al., 2008; Miller et al., 2011). Recently, ranaviral disease in amphibians was placed on the World Organization for Animal Health list of reportable diseases. Disease events in amphibians are often clustered into local epizootics, with significant impact on the local population (Green et al., 2002). These epizootics have been scattered across numerous habitats and landscapes in the US; however, disease predictability has not been successful. Furthermore, there have been increasingly more reports and cases in chelonians, and specifically box turtles, in the US (De Voe et al., 2004; Allender et al., 2006; Johnson et al., 2008).

Diagnostic assays that are validated and optimized to detect the presence of pathogens in certain species are key to characterizing the disease and understanding disease ecology. Quantitative PCR has not been developed previously for detection of FV3-like virus in turtles. Its use would provide much greater sensitivity in detection of FV3-like virus as compared with conventional PCR, and additionally would allow the quantitation of virus levels within specific tissues. Identifying clinical samples with the highest viral load will allow for more efficient sampling methods that target those tissues and potentially direct therapy toward those sites. Additionally, the greater sensitivity of qPCR will allow for the detection of ranavirus in animals with lower titers of virus, as might be seen in early or subclinical infections. In studies investigating iridoviruses in fish, TaqMan real-time PCR was shown to be 100 times more sensitive than conventional PCR, and crucial for the identification of subclinical disease states (Getchell et al., 2007; Pallister et al., 2007). Animals with subclinical infections may serve as important carriers or reservoirs for infectious disease; therefore, it is critical to develop assays capable of detecting the pathogen in these animals. To date, no such assay has been reported for ranavirus quantification in free-ranging or captive chelonians.

The purpose of this study was to develop and evaluate diagnostic methods for characterizing an emerging pathogen, FV3-like virus (genera *Ranavirus*, Family *Iridoviridae*) in box turtles. The hypotheses tested in this study were that a qPCR TaqMan based assay would be both sensitive and specific for characterizing FV3-like virus in box turtles. This is essential when considering the application of these assays to additional free-ranging populations and/or experimental models. Furthermore, it allows for evaluating potential climatic and environmental impacts of the disease, treatment, and management options.

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2. Materials and methods

2.1. DNA extraction

Whole blood and an oral swab were collected from a positive eastern box turtle that presented to the University of Tennessee (Knoxville, TN, USA) wildlife clinic in 2007. DNA was extracted following manufacturer's instructions (QIAamp DNA blood mini kit, Qiagen, Valencia, CA). Concentrations and purity of DNA were determined using a spectrophotometer (Nanodrop spectrophotometer, Thermo Scientific, Wilmington, DE).

2.2. Conventional PCR, sequencing, and cloning

Conventional PCR was performed using developed previously primers (sense: 5′-GACTTGGCCACTTATGAC-3′ and antisense: 5′-GTCTCTGGAGAAGAAGAA-3′) (MCP 4) targeting a portion of the major capsid protein (Mao et al., 1997). Products were sequenced in both directions using the University of Illinois Core DNA Sequencing Facility (Urbana, IL, USA) and compared to known sequences in GenBank using TBLASTX. For cloning, the PCR product (531 bp) from the MCP 4 primers was then cloned in *Escherichia coli* (TOPO TA Cloning® kit, Invitrogen, Carlsbad, CA). The cloning product was verified through sequencing in both directions. Plasmids were linearized with EcoR1, purified (QIAfilter plasmid Maxi kit, Qiagen, Valencia, CA), and quantified using spectrophotometry. Ten-fold serial dilutions of linearized plasmids were made from 10.0×10^2 ng/μl to 10×10^{-8} ng/μl. Viral genome (DNA) copy number was calculated using the following formula:

$$\#\text{copies}/\mu\text{L} = \frac{(\text{ng DNA of plasmid + clone}/\mu\text{L})(6.022 \times 10^{23}\ \text{copies/mol})}{(\text{bp length})(1 \times 10^9 \text{ng/g})(650\ \text{g/mol of bp})}$$

The final copy number for 10-fold serial dilutions ranged from 5.29×10^9 to 5.29×10^1 viral copies per reaction.

2.3. Real time qPCR assay

A primer/probe assay for a TaqMan-MGB (TaqMan® primers, FAM dye labeled, Applied Biosystems, Carslbad, CA) based qPCR assay were designed using a commercial software program (Primer Express®, Applied Biosystems, Carlsbad, CA) based on published sequences of the major capsid protein of FV3 (Mao et al., 1997). TaqMan assay was performed using forward (AACGCCGACCGAAAACTG), reverse (GCTGCCAAGATGTCGGGTAA), and probe (CCGGCTTTCGGGC) targeting a 54 bp segment of the major capsid protein (MCP) of frog virus 3. Real-time qPCR assays were performed using a real-time PCR thermocycler (7500 ABI real-time PCR System, Applied Biosystems, Carlsbad, CA) and data was analyzed using associated software (Sequence Detection Software v2.05, Applied Biosystems, Carlsbad, CA). Each reaction contained 12.5 μl of 2× TaqMan Platinum PCR Supermix-UDG with ROX (TaqMan Platinum PCR Supermix-UDG with ROX, Invitrogen, Carlsbad, CA), 1.25 μl TaqMan primer-probe, 2.5 μl turtle-derived FV3 dilution, and water to a final concentration of 25 μl. Cycling parameters were as follows: 1 cycle at 50 °C for 2 min followed by 95 °C for 10 min, then 40 cycles at 95 °C for 15 s and 60 °C for 60 s, and a final cycle of 72 °C for 10 min.

2.4. Standard curve, specificity, and sensitivity

To determine the sensitivity, assays were performed in three technical repeats on dilutions of turtle-derived positive control plasmid of FV3 MCP DNA (5.29×10^9–5.29×10^1 copies/rxn) within a single run. Standard curves were generated using the cycle threshold values of the positive control plasmid dilutions. Intra-assay variation was determined for both assays by calculating the mean C_t values, standard deviations, and coefficient of variations separately for each control plasmid DNA dilution. Efficiency curves of the dilutions were performed in uninfected cell culture lysates (spiked with plasmid dilutions), infected cell lysates, and turtle whole blood extracts from a positive sample.

2.5. Box turtle samples

Thirty-one eastern box turtles that were presented to the University of Tennessee College of Veterinary Medicine Wildlife clinic were examined during March through October 2007. Whole blood and swabs of the oral cavity were collected as described previously (Allender et al., 2011). The University of Tennessee Institutional Animal Use and Care Committee approved all animal use (protocol 1864). Whole blood and oral swab extracts of samples from animals with unknown disease status were evaluated. Results for detection were compared to published conventional PCR results for the same samples (Allender et al., 2011).

2.6. Statistical analysis

The quantity of ranavirus target DNA in infected whole blood and oral swabs was determined using a standard curve method. The copy number of the target DNA was determined from the standard curve generated with 10-fold dilutions of the positive control plasmid that contained the target sequence of the respective qPCR assay.

Copy numbers were tabulated and evaluated for normality using the Shapiro–Wilk test. Mean, median, standard deviation, 95% confidence interval, and 10–90% percentiles were determined for positive cases (copy number) for each assay. The Mann–Whitney *U* test was used to evaluate between assay differences. The prevalence of ranavirus was determined for each assay (categorical variable assigned; 1 = positive, 0 = negative). Exact 95% binomial confidence intervals were determined for all proportions. Level of agreement (kappa) was determined between both the real-time PCR assays and the conventional PCR reported previously based on prevalence. All statistical analysis was performed using statistical software (IBM SPSS Statistics 19, Chicago, IL).

3. Results

3.1. Conventional PCR, standard curve, reproducibility

Conventional PCR was performed to evaluate turtle-derived FV3-like virus dilutions using MCP 4 (Mao et al., 1997). The TaqMan primer set was designed to detect a 54 bp length gene segments of a conserved portion of the MCP gene of FV3.

Serial 10-fold dilutions of positive control plasmids were assayed using TaqMan assay and standard curves were generated based on C_t values (Fig. 1). The linear range for TaqMan was between 5.29×10^9 and 5.29×10^4 viral copies with an R^2 of 0.999 (slope = −3.277) (Fig. 1). Efficiency curves carried out on spiked-uninfected cell lysates (controlled for total DNA per rxn), infected cell lysates, and turtle whole blood extracts performed the equally well (data not shown). The amplification plots of TaqMan qPCR are presented in Fig. 2.

The intra- and inter-assay reproducibility was evaluated for the serial dilutions of the control plasmids (Table 1). The intra-assay CVs for TaqMan were between 0.04 and 0.24%. The inter-assay CVs were 0.14–0.3%, respectively. These results indicate high reproducibility between assays at all dilutions. The dynamic range for qPCR assays was from 5.29×10^9 to 5.29×10^1.

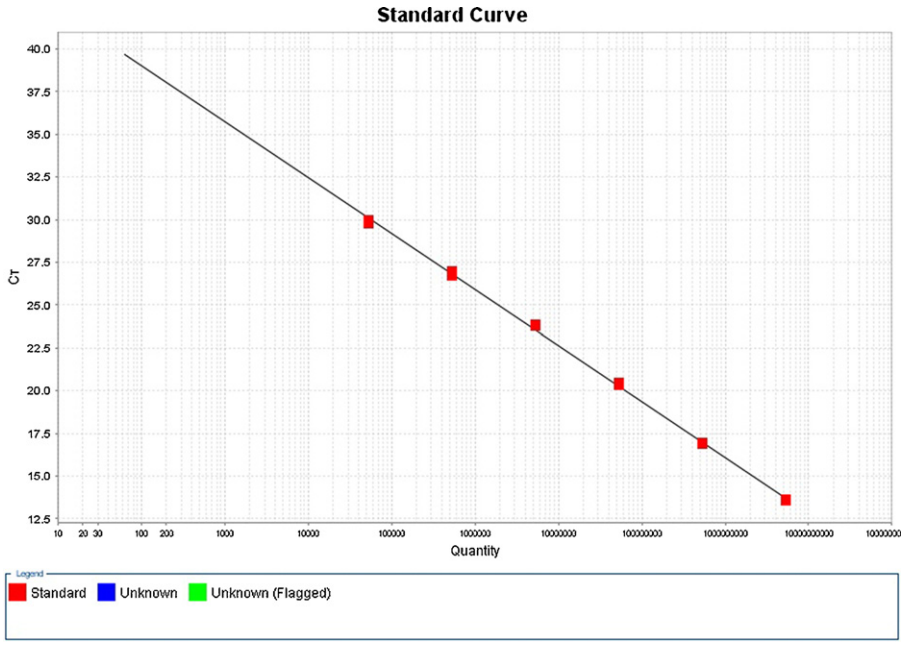

Fig. 1. Standard curve for a TaqMan probe-based primers obtained with 10-fold serial dilutions from 5.29×10^9 to 5.29×10^4 viral copies per reaction. The graph is plotted against a logarithmic concentration of the serial dilutions.

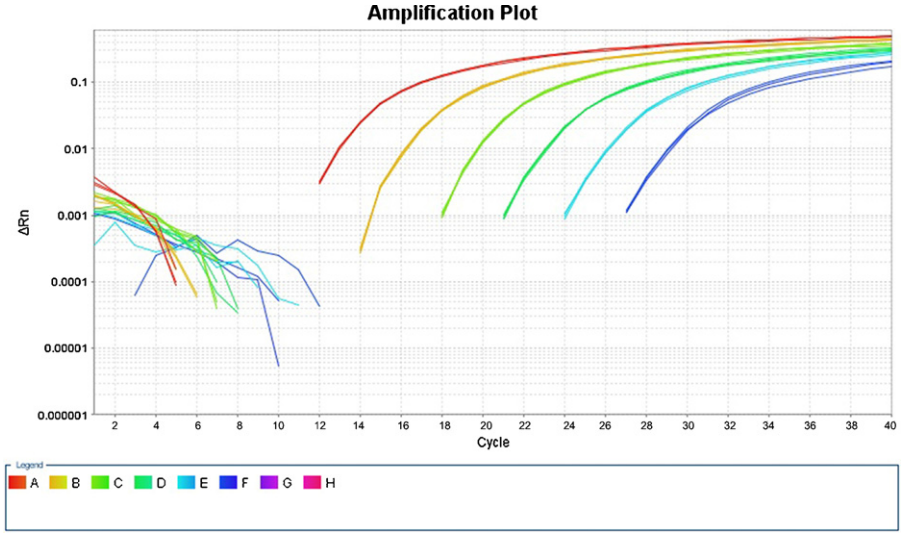

Fig. 2. Amplification plots for the standard curve for a TaqMan probe-based primers obtained with 10-fold serial dilutions from 5.29×10^9 to 5.29×10^4 viral copies per reaction.

Table 1
Intra- and inter-assay variability of a TaqMan qPCR assays detecting frog virus 3 major capsid protein.

	Intra-assay			Inter-assay		
Viral Copy TaqMan	CT mean	CT SD	CV	CT mean	CT SD	CV
5,290,000,000	13.14	0.02	0.14%	13.14	0.03	0.19%
529,000,000	16.45	0.04	0.20%	16.45	0.04	0.23%
52,900,000	19.93	0.03	0.14%	19.94	0.03	0.14%
5,290,000	23.31	0.04	0.14%	23.30	0.04	0.16%
529,000	26.34	0.07	0.23%	26.30	0.09	0.30%
52,900	29.36	0.08	0.24%	29.33	0.07	0.22%
5290	30.16	0.02	0.04%	30.14	0.05	0.15%
529	31.24	0.05	0.14%	31.17	0.09	0.26%
52	31.80	0.01	0.03%	31.85	0.07	0.19%
NTC	37.94	1.05	2.26%	37.94	1.05	2.26%

3.2. Box turtle samples

Twenty-nine blood samples and thirty oral swabs were collected routinely from turtles presented to the University of Tennessee from March through October 2007. Mean quantity of DNA recovered from blood samples measured through spectrophotometry was 726 ng/μl (95% CI: 550.38–902.05) and mean purity A260/280 was 1.85 (95%CI: 1.82–1.88). Median quantity of DNA recovered from swabs was 19.42 ng/μl (10–90%: 3.22–57.06) and A260/280 was 1.81 (10–90%: 1.20–2.05). Conventional PCR assays reported previously a 3% ($n = 1$; 95% CI: 0–9) prevalence rate for these samples (Allender et al., 2011). Quantitative PCR determined the presence of FV3-like virus in blood was 3% ($n = 1$; 95% CI: 0–9). However, prevalence of FV3-like virus in the oral swab samples using TaqMan was 6% ($n = 2$; 95% CI: 0–14). Mean viral quantity in blood for the single animal that was positive using TaqMan was

1.72×10^8. The mean viral copies in the swab sample from the same individual was 6.48×10^8. The number of viral copies in the other swab sample that was positive (no corresponding positive blood sample) was 4.8×10^3. Level of agreement between was substantial between conventional PCR and TaqMan qPCR ($\kappa = 0.651$).

4. Discussion

Ranaviral disease is one of only two amphibian diseases listed as reportable to the World Organization for Animal Health, but not yet reportable for reptiles. This is in part due the mass mortality events that have affected amphibian populations worldwide and the deficiency of data in reptile taxa (Miller et al., 2011). Despite its listing and severity of disease outbreaks, the epidemiology of this virus in amphibians, and hence reptiles, is not completely understood. Future and current epidemiologic surveys that determine the extent of disease and species range depend on diagnostic assays. The current study developed a quantitative PCR assay that allows detection of FV3-like virus in levels up to 1000 viral copies below conventional PCR based on dynamic range and levels of detection. The greater sensitivity may provide the opportunity to potentially detect early, subclinical, or reservoir states of the disease, which are crucial factors, that are currently unknown, but needed to characterize the epidemiology of ranavirus.

The level of detection was lower than the linear range of FV3-like virus copies for the assay. This indicated that the linear range was more reliable for quantification of higher viral copies than lower viral copies. It is possible that primer concentrations need to be optimized for low viral copy numbers, resulting in a separate protocol based on viral copy and should be pursued in future studies. These future studies should lower primer concentrations in assays of with less than 5.29×10^4 viral copies to establish a linear range that is more reliable for detection and quantification of low viral copy numbers. In the absence of that optimization, the developed assay is reliable for absolute quantitation of ranaviral DNA, with greater than 5.29×10^4 viral copies and relative quantitation for the entire dynamic range. Ultimately, the samples with viral copies below 5.29×10^4 are likely not biologically significant for this disease in this species, as viral quantity is either not detectable or above 10^4 in diseased animals (Allender, unpub. data)

TaqMan assays utilize a third selection step (probe), which provides for greater specificity not seen with other qPCR assays. The TaqMan assay is more stringent in requiring the hybridization of a probe to target a sequence between the PCR primers. Its degradation during PCR amplification is responsible for emitting a fluorescent signal. This additional step provides for a higher specificity when testing clinical samples. The assay had highly reproducible results with intra- and inter-assay variability coefficient of variation of less than 5%. The primers were designed to be specific for a segment of the major capsid protein gene that previous studies have targeted with conventional PCR (Johnson et al., 2008). It is highly conserved among various ranaviruses (Marsh et al., 2002; Jancovich et al., 2005; Liu et al., 2007; Johnson et al., 2008; Jancovich et al., 2010). While this assay was developed for use in outbreaks involving turtles and only FV3 or FV3-like viruses have been associated with those occurrences, future studies should evaluate the specificity of this assay with other similar ranaviruses to determine its application to diagnose infections involving other classes of animals.

DNA from 29 blood samples and thirty oral swabs were screened for FV3-like virus. These samples were selected because they had previously been screened using conventional PCR (Green et al., 2002). The results indicate that the TaqMan qPCR assay developed in this study was able to only identify a single additional positive oral swab sample, which was also identified using conventional PCR (Allender et al., 2011). This may signify that this population of turtles indeed had a low prevalence of FV3-like virus (not infected), were not shedding ranavirus in the sample, or did not have enough viral copies to return a positive result. The dual testing (conventional and qPCR methods) strategy used evaluating the two assays suggest that indeed the prevalence of infection was low in this population of box turtles. The epidemiology of ranavirus in turtles is not known, and therefore it is possible that when a susceptible individual is exposed to the virus, it either replicates to a level above 529,000 viral copies (conventional PCR level of detection) or does not replicate well. This would explain the results of conventional PCR and qPCR being similar. Regardless of the viral copies present in a clinical animal (as represented by these samples), early and subclinical detection should provide a valuable tool for control, monitoring, and management of this disease. However, specific transmission studies are needed to determine whether a subclinical or carrier state even exists in chelonians. In that event, this use of a highly sensitive qPCR assay will be needed. To further elucidate the sensitivity of this assays compared with conventional PCR, it will be important to apply the qPCR assay in a set of experimental samples where the prevalence of disease is high. As such, the quantitative assays reported here may be more useful in detecting and tracking the persistence and development of disease as once the animals present clinically, they are likely overwhelmed with similar viral loads.

Concentrations of DNA, measured by spectrophotometry, were greater from extracted whole blood samples than oral swab samples. This is not surprising, as whole blood of reptiles contains nucleated red blood cells, which leads to significantly more DNA. Swab samples mostly contain epithelial cells in fewer numbers and therefore less DNA. This difference may not be significant diagnostically as viral DNA concentrations are not necessarily increased in whole blood samples, and furthermore the increase in host DNA may lead to reduced efficiency during PCR assays. Therefore, the assay was validated in cell culture lysates and in whole blood dilutions in addition to plasmid dilutions. These validation conditions performed equally well as plasmid dilutions and thus can be concluded that there is not likely a reduced efficiency despite higher overall DNA concentrations in whole blood. Additionally, the assays are not based on a uniform amount of isolated material, therefore, blood should be normalized based on milliliter of blood. There were too few positive turtle samples to evaluate if higher DNA concentrations correlated with higher viral load, but future studies should investigate this correlation. Purity of DNA, determined by A260/A280 ratio, was within the ideal range (1.7–1.9) based on extraction guidelines for both whole blood and swab samples. However, the ratio was non-normally distributed for swab samples and the range was greater than whole blood samples. When the purity of the sample is outside the ideal range, it can lead to a decrease in assay efficiency or an increase in non-specific parameters and inaccurate results. Therefore, purity should be evaluated prior to qPCR assay for all swab samples and samples outside the ideal range should be further purified before assaying.

The current study demonstrated that a TaqMan assay is reliable, specific, and sensitive for the detection of a gene segment of the MCP of FV3-like virus. The TaqMan assay has low variability and was not likely to produce a non-specific product and therefore is the recommended assay for turtle FV3-like virus surveys. This assay can be used as a tool in the conservation of turtles by identifying emerging and ongoing outbreaks. This assay is more sensitive in detecting fewer viral copies than conventional PCR, thereby allowing for early, subclinical, or reservoir status detection if those states exist in turtles. As ranavirus continues to emerge as a major threat to reptiles and amphibians, it is critical that early and accurate identification of epidemics occur.

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