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Development and validation of quantitative PCR for detection of *Terrapene* herpesvirus 1 utilizing free-ranging eastern box turtles (*Terrapene carolina carolina*)

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A B S T R A C T

Diseases that affect the upper respiratory tract (URT) in chelonians have been well described as a significant contributor of morbidity and mortality. Specifically, herpesviruses are common pathogens in captive chelonians worldwide, but their importance on free-ranging populations is less well known. Historical methods for the diagnosis of herpesvirus infections include virus isolation and conventional PCR. Real-time PCR has become an essential tool for detection and quantitation of many pathogens, but has not yet been developed for herpesviruses in box turtles. Two quantitative real-time TaqMan PCR assays, TerHV58 and TerHV64, were developed targeting the DNA polymerase gene of *Terrapene* herpesvirus 1 (TerHV1). The assay detected a viral DNA segment cloned within a plasmid with 10-fold serial dilutions from 1.04×10^7 to 1.04×10^1 viral copies per reaction. Even though both primers had acceptable levels of efficiency and variation, TerHV58 was utilized to test clinical samples based on less variation and increased efficiency. This assay detected as few as 10 viral copies per reaction and should be utilized in free-ranging and captive box turtles to aid in the characterization of the epidemiology of this disease.

1. Introduction

Viruses from the order *Herpesvirales* are large, icosahedral, enveloped, double stranded, DNA viruses (McGeoch and Gatherer, 2005; Wellehan et al., 2004; Sim et al., 2015). This order is divided into three families, *Herpesviridae, Alloherpesviridae, and Malacoherpesviridae* (Davison, 2010). The family *Herpesviridae* is further divided into three subfamilies, *Alphaherpesvirinae, Betaherpesvirinae,* and *Gammaherpesvirinae* encompassing approximately 90 different species (Davison, 2010; ICTV, 2014). Currently, chelonian herpesviruses, in the genus *Scutavirus*, have DNA sequences most homologous to viruses in the alphaherpesvirus family (Bicknese et al., 2010; Greenblatt et al., 2005; Ossiboff et al., 2015a,b; Herbst et al., 2004; Origgi et al., 2015).

As has been seen in mammals and birds, several chelonian herpesviruses have been associated with clinical signs (Salinas et al., 2011; Stacy et al., 2008; Pettan-Brewer et al., 1996; Johnson et al., 2005). In tortoises, this virus has a predilection for mucoepithelial cells, commonly associated with rhinitis, conjunctivitis, stomatitis and glossitis (Salinas et al., 2011; Pettan-Brewer et al., 1996; Johnson et al., 2005; Ossiboff et al., 2015a). Captive Greek tortoises inoculated with herpesvirus developed necrotizing stomatitis and rhinitis (Origgi et al., 2004). A captive desert tortoise diagnosed with a herpesvirus infection exhibited anorexia, lethargy, and caseous glossal plaques (Johnson et al., 2005). In aquatic turtles, herpesvirus infections have been linked to skin disease (Rebell et al., 1975; Cowan et al., 2015; Yonkers et al., 2015). Marine turtles affected with Gray Patch Disease, associated with Chelonid herpesvirus 1, develop circular gray skin lesions (Rebell et al., 1975). The most common clinical sign seen in marine turtles, with a herpesvirus infection due to Chelonid herpesvirus 5, is development of neoplastic fibropapillomas (Jacobson et al., 1991; Quackenbush et al., 2001; Chaloupka et al., 2009). Chelonid herpesvirus 6 has been associated with lung-eye-trachea disease in marine turtles (Curry et al., 2000; Coberely et al., 2002; Jacobson et al., 1986). However, some reported herpesvirus infections are fatal before any premonitory signs are noted (Harper et al., 1982; Jungwirth et al., 2014).

Reports of box turtle herpesvirus are scarce. The presence of a unique virus has been demonstrated to occur in three different col-

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lections (Sim et al., 2015; Yonkers et al., 2015). An eastern box turtle (*Terrapene carolina carolina*) which emerged early from brumation with lethargy, dehydration, and dyspnea died a week later and was diagnosed with herpesvirus on histopathology and PCR (Sim et al., 2015). A separate collection of turtles infected with ranavirus was opportunistically tested for herpesvirus via PCR and was found to have a 58% prevalence (Sim et al., 2015). A free ranging eastern box turtle with a papillomatous growth was diagnosed with a novel herpesvirus by nested PCR (Yonkers et al., 2015). Herpesvirus was diagnosed by PCR in a captive Australian Krefft's river turtle (Emydura macquarii krefftii), which presented with severe proliferative and ulcerative skin and shell lesions (Cowan et al., 2015). A captive, juvenile, female northern map turtle (*Graptemys geographica*) was diagnosed with herpesviral pneumonia, which was confirmed by PCR, after exhibiting weakness and nasal discharge (Ossiboff et al., 2015a). Three novel herpesviruses were identified in free-ranging bog turtles (*Glyptemys muhlenbergii*), wood turtles (*G. muhlenbergii*), and spotted turtles (*Clemmys guttata*) by PCR (Ossiboff et al., 2015b). Additionally, two clinically healthy West African mud turtles (*Pelusios castaneus*) were diagnosed with a novel herpesvirus infection by PCR (Marschang et al., 2015). Nonetheless, the epidemiology in free-ranging eastern box turtle populations is still unknown.

Several diagnostic methods have been used to diagnose herpesvirus infections in chelonians, including virus isolation and conventional PCR. Quantitative PCR (qPCR) has not been previously developed for the detection of *Terrapene* herpesvirus 1 (TerHV1) in chelonians and would provide increased sensitivity as compared to conventional PCR. This method would allow quantitation of viral copy numbers. Furthermore, it may allow detection of carriers with low levels of virus particles. Animals with subclinical infections may serve as important carriers or reservoirs for infectious disease; therefore, it is crucial for assay development to be capable of detecting pathogens in these animals. The quantification of viral copy numbers may also allow for therapeutic monitoring of infections. To date, no such assay has been reported for herpesvirus quantification in free-ranging or captive box turtles.

The purpose of this study was to develop and evaluate diagnostic methods for detecting TerHV1 in eastern box turtles. The hypothesis tested in this study was that a Taqman qPCR assay would be sensitive for detecting *Terrapene* herpesvirus 1 (Order *Herpesvirales*, Family *Herpesviridae*, Subfamily *Alphaherpesvirinae*, Genus *Scutavirus*) in eastern box turtles. This quantitative method will be useful for early detection and disease monitoring in both captive and free-ranging chelonian populations.

2. Materials and methods

2.1. Conventional PCR, sequencing, and cloning

An oral swab was collected from a suspect positive free-ranging eastern box turtle in Tennessee determined by associated clinical signs, such as rhinitis, glossitis, or stomatitis. DNA was extracted according to the manufacturer's instructions (QIAamp DNA blood mini kit, Qiagen, Valencia, California, USA). Nested PCR was performed as previously described for herpesvirus detection (VanDevanter et al., 1996) and later documented for detection of herpesviruses in chelonians (Marschang et al., 2006). Primary PCR contained two sense (DFA, 5′-GAYTTYGCNAGYYTNTAYCC-3′; and ILK, 5′-TCCTGGACAAGCAGCARNYSGCNMTNAA-3′) and one anti-sense (KG1, 5′-GTCTTGCTCACCAGNTCNACNCCYTT-3′) primers. Secondary PCR assays were performed with sense (TGV, 5′-TGTAACTCGGTGTAYGGNTTYACNGGNGT-3′) and anti-sense (IYG, 5′-CACAGAGTCCGTRTCNCCRTADAT-3′) primers targeting a herpesvirus-consensus region of the DNA polymerase gene. Reactions were performed in 25 μL total volume using Premix taq (Takara Taq version) containing hot start Taq (TaKaRa Bio Inc.). Five μl of template DNA from the first step was used in the second reaction. Feline herpesvirus 1 and canine herpesvirus 1 were used as positive controls, while DNase/RNase-free water was used as a negative control. PCR products were resolved by electrophoresis in 1.4% agarose gels. Products were sequenced in both directions using the Molecular Biology Resource Facility at the University of Tennessee and compared to known sequences in GenBank using TBLASTX. Phylogenetic analysis of herpesvirus sequences obtained from clinical samples and the literature (Sim et al., 2015; KJ004665) were used to design a consensus 480 base pair gene segment (Integrated DNA Technologies, Coralville, Iowa) that was then cloned in *Escherichia coli* (TOPO TA Cloning® kit, Invitrogen, Carlsbad, CA). The cloning products were verified through sequencing in both directions (Keck Biotechnology Center at the University of Illinois). Plasmids were linearized with BamH1 (Clontech Laboratories, Mountain View, CA), purified (QIAfilter plasmid Maxi kit, Qiagen, Valencia, CA), and quantified using a spectrophotometer (Nanodrop spectrophotometer, Thermo Scientific, Wilmington, DE). Ten-fold serial dilutions of linearized plasmids were made from 1.04×10^9 ng/μl to $1.04 \times 10^{\circ}$ ng/μl. Viral genome (DNA) copy number was calculated using the following formula:

$$\#\text{copies}/\mu\text{L} = \frac{\left(\text{ng DNA of plasmid} \pm \text{clone}/\mu\text{L}\right)\left(6.022 \times 10^{23}\text{copies/mol}\right)}{\left(\text{base per length}\right)\left(1 \times 10^{9}\text{ng/g}\right)\left(650\text{g/mol of base pair}\right)}$$

The final copy number for ten-fold serial dilutions ranged from 1.04×10^7 to 1.04×10^1 viral copies per reaction.

2.2. Real time qPCR assay

Two TaqMan-MGB (Taqman® primers, FAM dye labeled, Applied Biosystems, Carslbad, CA) qPCR assays, TerHV58 and TerHV64, for TaqMan-MGB (Taqman® primers, FAM dye labeled, Applied Biosystems, Carslbad, CA) qPCR assays were designed using a commercial software program (Primer Express®, Applied Biosystems, Carlsbad, CA) based on the sequence of the TerHV1 DNA polymerase gene segment in our clone. The TaqMan with assay TerHV58 was performed using forward (5′-TCTATTGGGCGAGCTGTTGAC-3′), reverse (5′-GAAAAGCCATACGCCTACGC-3′), and probe (5′-ACTGGCTGGCCTTG-3′), targeting a 58 base pair segment of the herpesvirus DNA polymerase gene. The TaqMan with assay TerHV64 was performed using forward (5′-GGCCACCCGAGATTATATCCA-3′) reverse (5′-TTCTGGCCGACTTCCCG-3′), and probe (5′-CCCATTGGAGCGAACGGGAAAAG-3′), targeting a 64 base pair segment of the herpesvirus DNA polymerase gene. 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 20x 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 plasmid dilution, and water to a final volume 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 seconds and 60 °C for 60 seconds, and a final cycle of 72 °C for 10 min.

2.3. Standard curve and sensitivity

To determine the sensitivity, assays were performed in four technical repeats on dilutions of turtle-derived positive control plasmid of TerHV1 DNA (1.04×10^7–1.04×10^1 copies/rxn) within a single run. Standard curves were generated using the cycle thresh-