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Short communication

Validation of a sensitive PCR assay for the detection of Chelonid fibropapilloma-associated herpesvirus in latent turtle infections

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ABSTRACT

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The Chelonid fibropapilloma-associated herpesvirus (CFPHV) is hypothesized to be the causative agent of fibropapillomatosis, a neoplastic disease in sea turtles, given its consistent detection by PCR in fibropapilloma tumours. CFPHV has also been detected recently by PCR in tissue samples from clinically healthy (non exhibiting fibropapilloma tumours) turtles, thus representing presumably latent infections of the pathogen. Given that template copy numbers of viruses in latent infections can be very low, extremely sensitive PCR assays are needed to optimize detection efficiency. In this study, efficiency of several PCR assays designed for CFPHV detection is explored and compared to a method published previously. The results show that adoption of a triplet set of singleplex PCR assays outperforms other methods, with an approximately 3-fold increase in detection success in comparison to the standard assay. Thus, a new assay for the detection of CFPHV DNA markers is presented, and adoption of its methodology is recommended in future CFPHV screens among sea turtles.

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1. Introduction

Fibropapillomatosis is a neoplastic disease in sea turtles characterized by cutaneous tumours, that is known to infect all sea turtle species across their global distribution (Ene et al., 2005; Herbst et al., 2004). Although multiple hypotheses have been proposed behind the causative agent of fibropapillomatosis, including chemical carcinogens, ultraviolet light, oncogenic viruses and metazoan parasites (Smith and Coates, 1938; Herbst and Klein, 1995; Casey et al., 1997; Herbst et al., 1998, 1999; Greenblatt et al., 2004), that accepted most widely is the Chelonid fibropapilloma-associated herpesvirus (CFPHV) (Herbst, 1994; Work et al., 2009). Previous studies have detected the presence of CFPHV DNA in fibropapilloma tumour biopsies, and viral DNA sequences were also recovered recently from normal tissue from fibropapillomatosis exhibiting animals (Ene et al., 2005; Page-Karjian et al., 2012). The detection of CFPHV in tumour biopsies is not surprising – herpesviruses are known to replicate in tumours and tissue of debilitated animals (Herbst et al., 1995), thus large concentrations of viral DNA are present. In contrast however, when herpesviruses are present as latent infections – that is in individuals that do not exhibit

clinical evidence of disease, the level of viral DNA in the tissues will be much lower, thus representing a detection challenge for methods used to assay for the virus' presence and prevalence.

There are several clinical techniques for detection of viral nucleic acid sequences in an infected host. Singleplex PCR assays targeting relatively short amplicons (short-range PCR) are among those used most commonly, and have been reported to be more sensitive for viral detection than real-time PCR assays, for example as proven in detecting white spot syndrome virus (WSSV) (Mrotzek et al., 2010), infectious hypodermal and hematopoietic necrosis virus (IHHNV) (Katzourakis and Gifford, 2010) and monodon baculovirus (MBV) in shrimp (Mrotzek et al., 2010); porcine reproductive and respiratory syndrome virus (PRRSV); transmissible gastroenteritis virus (TGEV), porcine respiratory coronavirus (PCRV) and pseudorabies virus (PRV) in pigs (Braun and Chase, 1999; Minarovits et al., 2006; Chentoufi et al., 2011) and various human papillomaviruses (Kleter et al., 1998; Morshed et al., 2008). In addition to conventional PCR, nested PCR assays have also been employed, following results suggest that they may increase detection sensitivity by 10,000-fold (Fox et al., 1995; Klein et al., 1998; Lu et al., 2000, 2003; Quackenbush et al., 2001).

In this study, the sensitivity of several alternate PCR assays designed to detect CFPHV DNA was compared, in order to provide a recommendation on a suitable method for assaying viral prevalence in both tumour and non-tumour samples of sea turtles. The assays include (i) a nested-PCR based assay published previously,

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Table 1

Singleplex PCR primers and nested primers used for DNA detection of the Chelonid fibropapilloma-associated herpesvirus (CFPHV) short markers.

Primer set	Targeted gene	Primer sequence (5'–3')	Length of amplification (nucleotide bases)
Nested primers			
<i>Previously tested nested primers Lu.et.UL30</i>			
Primary: Lu.et.UL30	Polymerase gene UL30	F: AGCATCATCCAGGCCACAATCT R: CGGCCAGTTCGGCGCTCGACCA	445
Secondary: Lu.et.UL30	Polymerase gene UL30	F: AGCATGTCCGCGCCTACGGTGGTGAC R: CTGCTGACCGACTGGCTGGC	206
<i>Designed in this study</i>			
Primary: UL18	Capsid protein gene UL18	F: CACCACGAGGGGAAAATGA R: TCAAATCCCCGTTCACTCG	717
Secondary: UL18	Capsid protein gene UL18	F: GTGGAACCCCGCCGGTAAT R: TGATCCGGCCGAGTAGCCGG	140
Primary: UL22	Glycoprotein H gene UL22	F: ACGGCGTTGGCTAGTGAATC R: GCAGTTCGGTACACCTCT	386
Secondary: UL22	Glycoprotein H gene UL22	F: AACGCCCTTCTCCGACCCATATT R: GCTGGGGAGCATCGTGCAA	179
Primary: UL27	Glycoprotein B gene UL27	F: TAACAAGAAAGAACC CGCGC R: ATTTTCCCGTCACTGCGCAA	352
Secondary: UL27	Glycoprotein B gene UL27	F: CTAGATACATACTGGCCTGCTCGTC R: GCCAGCGACCATCCGGAG	143
Singleplex primers (designed in this study)			
UL18	Capsid protein gene UL18	F: GTGGAACCCCGCCGGTAAT R: TGATCCGGCCGAGTAGCCGG	140
UL22	Glycoprotein H gene UL22	F: AACGCCCTTCTCCGACCCATATT R: GCTGGGGAGCATCGTGCAA	179
UL27	Glycoprotein B gene UL27	F: CTAGATACATACTGGCCTGCTCGTC R: GCCAGCGACCATCCGGAG	143

(ii) a nested PCR-based approach designed here to target short segments of three genes of the CFPHV, and (iii) three new singleplex PCR primer sets targeting the same three viral genes.

2. Materials and methods

DNA extracts from 132 putatively CFPHV positive turtle samples (detailed information of sample location and type of tissue can be found in supplementary Table S1), sampled as part of a recent prevalence study (Alfaro-Núñez et al. in review, supplementary data S2) were used to cross-compare the performance of several new PCR-based assays designed to detect CFPHV. These samples represent both active (FP tumour) and latent (non-tumour) CFPHV infections. Non-tumour samples are defined as any other type of tissue with macroscopic absence of tumour and taken from either fibropapillomatosis exhibiting or clinically healthy not exhibiting turtles. A published previously (VanDevanter et al., 1996; Lu et al., 2000) nested approach, hereafter referred to as Lu.et.UL30, that was designed to target the CFPHV polymerase (*pol*) gene UL30, was used as baseline control against which new approaches were compared

as described in Table 1. It can be challenging to PCR amplify targets within latent viral infections that exhibit potentially sequence variation at primer binding sites, and/or are present at low copy numbers. Two new detection assays, one based around singleplex PCR, the other around nested PCR were designed. Both involve three independent PCR assays that utilize primer sets that bind to highly conserved regions for three different genes in the CFPHV genome (Glycoprotein B gene UL27, Glycoprotein H gene UL22 and Major capsid protein gene UL18-UL19) to maximize chance of detection (Table 1). Primers were designed using the software PRIMER3 (v.0.4.0) implemented in GENEIOUS PRO 7.0.6 (Biomatters, New Zealand).

The singleplex PCR assays consists of one unique primer set per marker, and target amplicons of 140 bp, 179 bp and 143 bp for the UL27, UL22 and UL18-UL19 marker respectively. The nested PCR assay consists of two primer sets per target region. Initially a primary PCR using 1 µl of DNA extract was PCR amplified for 39 cycles. These products were purified using the QIAquick PCR Purification Kit (Qiagen, USA) following the instructions of the manufacturer, of which 2 µl was used as template for secondary (nested) PCR.

Table 2

Overall performance of nested (both previously published and designed in this study) and new singleplex PCR primer sets test in a dataset of 132 putatively CFPHV DNA extracts.

Primer sets	Individual primer set performance		Overall performance	
	Total positive PCR viral detections per individual primer set	Proportion of viral detection in total infected cases ($n = 132$)	Overall viral detection per PCR assay method	Proportion of viral detection per PCR assay method in total infected cases ($n = 132$)
<i>Nested primers</i>				
Lu.et.(UL30)	22	16.7%	Previously published Lu.et.(UL30)	16.7%
UL18	25	18.9%	22	
UL22	37	28.0%	Combined nested	33.3%
UL27	27	20.5%	UL18 + UL22 + UL27	
<i>Singleplex primers</i>				
UL18	58	43.9%	44	
UL22	93	70.5%	Combined singleplex	100%
UL27	76	57.6%	UL18 + UL22 + UL27	
			132	

The target sizes and primer sequences of the primary and nested reaction are listed in Table 1. The products of all amplifications (singleplex, nested-primary and nested-secondary) were visualized on 2% agarose gels. In total therefore, each of the 132 DNA extracts were tested in 11 distinct PCR's for viral detection.

All PCR amplifications were performed in 25 µl reactions using the enzyme AmpliTaq Gold® 360 Master Mix (Life Technologies, USA). Each reaction contained 1× PCR buffer, 1× mM MgSO₄, 0.2 mM each primer, 0.2 mM dNTP mixture and 0.2 µl polymerase. Each PCR was cycled under the following conditions: initialization 94 °C for 4 min, 39 cycles of (94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s), final extension 72 °C for 7 min.

3. Results

When viewed as a set, the singleplex assay detected virus in 100% of the extracts, in comparison to only 33% of samples using the nested assays. Both outperformed the control-nested assay *Lu.et.UL30*, which detected viral sequences in only 17% of the cases as shown in Table 2 and in detail per each DNA extract in supplementary Table S1.

The detection sensitivity for CFPHV DNA varies considerably by PCR assay. Among the individual singleplex assays UL22 yielded positive results for 70.5% of the samples, UL27 for 57.6% and UL18 for 43.9% (Table 2). All singleplex primer sets outperform nested PCR assays, with nested UL22 being the highest in viral detection (28%). Moreover, there were only three positive viral samples that were detected uniquely positive by only one nested and singleplex primer sets. All other nested positive detections were confirmed simultaneously by at least two of the singleplex primers (Table S1).

4. Discussion

The specificity of all amplicons to CFPHV was confirmed through Sanger sequencing (Alfaro-Núñez et al. in review, supplementary data S2). The control nested assay was implemented following the conditions described by Lu et al. (2000), who applied originally this to a Hawaiian green turtle fibropapillomatosis test population. Subsequently it has been applied for detection of CFPHV in greens and loggerheads turtles from Florida (Klein et al., 1998) and in two green turtle populations in Puerto Rico (Page-Karjian et al., 2012), with success rates that were higher over a mixture of tumour and non-tumour samples than reported here. The dataset presented in this study was derived from tumour and non-tumour samples from fibropapillomatosis exhibiting green turtles, and healthy turtles (thus not fibropapillomatosis exhibiting) from five turtle species worldwide creating a bigger challenge for the detection method applied (supplementary Table S1). The variety of tissue material used for the DNA extracts may explain the difference in detection when using control-nested assay *Lu.et.UL30* with comparing other studies.

It is hypothesized that precision of detection may be correlated to the initial viral DNA concentration and DNA fragment sizes available in the sample; as well as the PCR chemistry to anneal to the targeted viral DNA. The potential role of viral concentration in PCR success is obvious. That of DNA fragment sizes is relevant for nested PCR, due to relatively larger size of the primary amplicon in relation to that of singleplex amplicons. Thus under any condition where DNA is degraded, the singleplex reactions will perform better.

5. Conclusion

In summary, the singleplex assay presented in this study represents a tool with improved sensitivity for use in studies on CFPHV detection and prevalence, and will be likely of particular use in

analyses where the viral concentration is hypothesized to be low. Thus, the validated method described will make possible to map out the occurrence of clinically and latent CFPHV infected turtles. As such, the method is likely to represent a competitive alternative to previous detection methods.

Competing interests

The authors declare that they have no competing interests.

Author's contributions

Alonzo Alfaro-Núñez carried out the molecular genetic assays, general data analysis, study design and drafted the manuscript; and M. Thomas P. Gilbert conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jviromet.2014.05.019>.

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